10/089501 10/129202

JC20 Rec'd PCT/PTO 29 MAR 2002

FORM PTO-1390 (Modified) (REV 5-93) U S DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE		ATTORNEY'S DOCKET NUMBER
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371		053466-0325
		U.S APPLICATION NO (If known, see 37 C.F.R. 1.5) Unassigned 10/089501
INTERNATIONAL APPLICATION NO. PCT/JP00/06802	INTERNATIONAL FILING DATE 29 September 2000	PRIORITY DATE CLAIMED 1 October 1999

TITLE OF INVENTION
PREVENTION AND TREATMENT OF BLOOD COAGULATION-RELATED DISEASES

APPLICANT(S) FOR DO/EO/US
Hiroyuki SAITO, Takehisa KITAZAWA, Kazutaka YOSHIHASHI, and Kunihiro HATTORI

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a FIRST submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371.
3. ☐ This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   - ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
   - ☒ has been transmitted by the International Bureau.
   - ☐ is not required, as the application was filed in the United States Receiving Office (RO/US)
6. ☒ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   - ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
   - ☐ have been transmitted by the International Bureau.
   - ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   - ☒ have not been made and will not be made.
8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).
11. ☐ Applicant claims small entity status under 37 CFR 1.27 .

Items 12. to 17. below concern other document(s) or information included:

12. ☒ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
13. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
14. ☒ A FIRST preliminary amendment.
    ☐ A SECOND or SUBSEQUENT preliminary amendment.
15. ☐ A substitute specification.
16. ☐ A change of power of attorney and/or address letter.
17. ☒ Other items or information: Application Data Sheet (4 pages), Sequence Listing (57 pages), Receipt in the Case of an Original Deposit with Translation (2 pages)

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JC13 Rec'd PCT/PTO 29 MAR 2002

U.S. APPLICATION NO. (If known, see 37 C.F.R. 1.5) Unassigned 10/089501	INTERNATIONAL APPLICATION NO PCT/JP00/06802	ATTORNEY'S DOCKET NUMBER 053466-0325

18. ☒ The following fees are submitted:	CALCULATIONS	PTO USE ONLY
Basic National Fee (37 CFR 1.492(a)(1)-(5): Search Report has been prepared by the EPO or JPO.....$890.00		
International preliminary examination fee paid to USPTO (37 CFR 1.482).....$710.00		
No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2) $740.00		
Neither international preliminary examination fee (37 CFR 1.482) nor International search fee (37 CFR 1.445(a)(2)) paid to USPTO $1,040.00		
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4) $100.00		
ENTER APPROPRIATE BASIC FEE AMOUNT =	$890.00	
Surcharge of $130.00 for furnishing the oath or declaration later than 20 Months from the earliest claimed priority date (37 CFR 1.492(e))		

Claims	Number Filed		Included in Basic Fee		Extra Claims		Rate		
Total Claims	53	-	20	=	33	×	$18.00	$594.00	
Independent Claims	6	-	3	=	3	×	$84.00	$252.00	
Multiple dependent claim(s) (if applicable)							$280.00		
TOTAL OF ABOVE CALCULATIONS =								$1736.00	
Reduction by ½ for filing by small entity, if applicable.								$0.00	
SUBTOTAL =								$1736.00	
Processing fee of $130.00 for furnishing English translation later the 20 months from the earliest claimed priority date (37 CFR 1.492(f). +									
TOTAL NATIONAL FEE =								$1736.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). $40.00 per property +									
TOTAL FEES ENCLOSED =								$1736.00	
							Amount to be: refunded	$	
							charged	$	

a. ☒ A check in the amount of $1736.00 to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. 19-0741 in the amount of $0.00 to the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 19-0741. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

Foley & Lardner
Customer Number: 22428

22428

PATENT TRADEMARK OFFICE

SIGNATURE

NAME HAROLD C. WEGNER

REGISTRATION NUMBER 25,258

Application Data Sheet

Application Information

Application Type::	Regular
Subject Matter::	Utility
Suggested classification::	
Suggested Group Art Unit::	
CD-ROM or CD-R?::	None
Computer Readable Form (CRF)?::	Yes
Number of copies of CRF::	1
Title::	PREVENTION AND TREATMENT OF BLOOD COAGULATION-RELATED DISEASES
Attorney Docket Number::	053466-0325
Request for Early Publication?::	No
Request for Non-Publication?::	No
Suggested Drawing Figure::	1
Total Drawing Sheets::	14
Small Entity?::	No
Petition included?::	No
Secrecy Order in Parent Appl.?::	No

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Representative Information

Representative Customer Number::	22428	

Domestic Priority Information

Application::	**Continuity Type::**	**Parent Application::**	**Parent Filing Date::**
This Application	National Stage of	PCT/JP00/06802	09/29/200

Foreign Priority Information

Country::	**Application number::**	**Filing Date::**	**Priority Claimed::**
Japan	11-281843	10/01/1999	Yes
Japan	11-282120	10/01/1999	Yes

Japan	11-282134	10/01/1999	Yes
Japan	11-282167	10/01/1999	Yes
Japan	11-282188	10/01/1999	Yes
Japan	11-282192	10/01/1999	Yes

Assignee Information

Assignee name:: CHUGAI SEIYAKU KABUSHIKI KAISHA

Atty. Dkt. No. 053466-0325

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Hiroyuki SAITO et al.

Title: PREVENTION AND TREATMENT OF DISEASES ASSOCIATED WITH BLOOD COAGULATION

Appl. No.: Unassigned

Filing Date: 03/29/2002

Examiner: Unassigned

Art Unit: Unassigned

PRELIMINARY AMENDMENT

Commissioner for Patents
Box PCT
Washington, D.C. 20231

Sir:

Prior to examination of the above-identified application, Applicants respectfully request that the above-identified application be amended as follows:

In the Claims:

In accordance with 37 C.F.R. §1.21, please substitute for claims 3, 5 – 8, 12 – 14, 16, 21 – 23, 25, 30 – 32, 34, 39 – 41, 43, 48 – 50, and 52 the following rewritten version of the same claims, as amended. The changes are shown explicitly in the attached "Marked-up Version of Amended Claims."

3. (Once Amended) The animal according to claim 1 wherein said animal cell is a mammalian cell.

5. (Once Amended) The animal according to claim 1 wherein saidm animal is a mouse.

6. (Once Amended) The animal according to claim 1 wherein said hypercoagulable state is indicated by at least one of the phenomena comprising an

increase in the plasma concentration of human tissue factor, a decrease in platelets, a decrease in fibrinogen, an increase in the concentration of soluble fibrin monomer complex, and an increase in the concentration of thrombin-antithrombin III complex.

7. (Once Amended) A method of generating the animal according to claim 1, wherein an animal cell to which the gene encoding human tissue factor (TF) or part thereof has been inserted and which is capable of expressing said gene is implanted to non-human animals and then an animal having a persistent hypercoagulable state is selected.

8. (Once Amended) A method of screening an anti-thrombotic agent which method comprises using the animal according to claim 1.

12. (Once Amended) The preventive or therapeutic agent according to claim 9 wherein said antibody is an altered antibody.

13. (Once Amended) The preventive or therapeutic agent according to claim 9 wherein said antibody is an altered antibody.

14. (Once Amended) The preventive or therapeutic agent according to claim 9 wherein said altered antibody is a chimereic antibody or a humanized antibody.

16. (Once Amended) The preventive or therapeutic agent according to claim 9 wherein said antibody is a modified antibody.

21. (Once Amended) The preventive or therapeutic agent according to claim 18 wherein said antibody is a recombinant antibody.

22. (Once Amended) The preventive or therapeutic agent according to claim 18 wherein said antibody is an altered antibody.

23. (Once Amended) The preventive or therapeutic agent according to claim 18 wherein said antibody is an altered antibody.

24. (Once Amended) The preventive or therapeutic agent according to claim 18 wherein said altered antibody is a chimereic antibody or a humanized antibody.

25. (Once Amended) The preventive or therapeutic agent according to claim 18 wherein said antibody is a modified antibody.

30. (Once Amended) The preventive or therapeutic agent according to claim 27 wherein said antibody is a recombinant antibody.

31. (Once Amended) The preventive or therapeutic agent according to claim 27 wherein said antibody is an altered antibody.

32. (Once Amended) The preventive or therapeutic agent according to claim 27 wherein said altered antibody is a chimeric antibody or a humanized antibody.

34. (Once Amended) The preventive or therapeutic agent according to claim 27 wherein said antibody is a modified antibody.

39. (Once Amended) The preventive or therapeutic agent according to claim 36 wherein said antibody is a recombinant antibody.

40. (Once Amended) The preventive or therapeutic agent according to claim 36 wherein said antibody is an altered antibody.

41. (Once Amended) The preventive or therapeutic agent according to claim 36 wherein said altered antibody is a chimeric antibody or a humanized antibody.

43. (Once Amended) The preventive or therapeutic agent according to claim 36 wherein said antibody is a modified antibody.

48. The preventive or therapeutic agent according to claim 45 wherein said antibody is a recombinant antibody.

49. (Once Amended) The preventive or therapeutic agent according to claim 45 wherein said antibody is an altered antibody.

50. (Once Amended) The preventive or therapeutic agent according to claim 45 wherein said altered antibody is a chimeric antibody or a humanized antibody.

52. (Once Amended) The preventive or therapeutic agent according to claim 43 wherein said antibody is a modified antibody.

REMARKS

Applicants respectfully request that the foregoing amendments be made prior to examination of the present application. The amendments are made to correct multiple dependencies and do not change the scope of the invention.

Respectfully submitted,

Date March 29, 2002

By _____

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Registration No. 25,258

Atty. Dkt. No. 053466-0325

MARKED UP VERSION OF AMENDED CLAIMS

3. (Once Amended) The animal according to claim 1 [or 2] wherein said animal cell is a mammalian cell.

5. (Once Amended) The animal according to [any one of claims 1 to 4] claim 1 wherein saidm animal is a mouse.

6. (Once Amended) The animal according to [any one of claims 1 to 5] claim 1 wherein said hypercoagulable state is indicated by at least one of the phenomena comprising an increase in the plasma concentration of human tissue factor, a decrease in platelets, a decrease in fibrinogen, an increase in the concentration of soluble fibrin monomer complex, and an increase in the concentration of thrombin-antithrombin III complex.

7. (Once Amended) A method of generating the animal according to [any one of claims 1 to 6] claim 1, wherein an animal cell to which the gene encoding human tissue factor (TF) or part thereof has been inserted and which is capable of expressing said gene is implanted to non-human animals and then an animal having a persistent hypercoagulable state is selected.

8. (Once Amended) A method of screening an anti-thrombotic agent which method comprises using the animal according to [any one of claims 1 to 6] claim 1.

12. (Once Amended) The preventive or therapeutic agent according to claim 9 [or 11] wherein said antibody is an altered antibody.

13. (Once Amended) The preventive or therapeutic agent according to claim 9 [or 12] wherein said antibody is an altered antibody.

14. (Once Amended) The preventive or therapeutic agent according to claim 9 [,12, or 13] wherein said altered antibody is a chimereic antibody or a humanized antibody.

16. (Once Amended) The preventive or therapeutic agent according to claim 9 [or any one of claims 12-15] wherein said antibody is a modified antibody.

21. (Once Amended) The preventive or therapeutic agent according to claim 18 [or 20] wherein said antibody is a recombinant antibody.

22. (Once Amended) The preventive or therapeutic agent according to claim 18 [or 21] wherein said antibody is an altered antibody.

23. (Once Amended) The preventive or therapeutic agent according to claim 18 [, 21, or 22] wherein said altered antibody is a chimereic antibody or a humanized antibody.

25. (Once Amended) The preventive or therapeutic agent according to claim 18 [or any one of claims 21-24] wherein said antibody is a modified antibody.

30. (Once Amended) The preventive or therapeutic agent according to claim 27 [or 29] wherein said antibody is a recombinant antibody.

31. (Once Amended) The preventive or therapeutic agent according to claim 27 [or 30] wherein said antibody is an altered antibody.

32. (Once Amended) The preventive or therapeutic agent according to claim 27 [, 30, or 31] wherein said altered antibody is a chimeric antibody or a humanized antibody.

34. (Once Amended) The preventive or therapeutic agent according to claim 27 [or any one of claims 30-33] wherein said antibody is a modified antibody.

39. (Once Amended) The preventive or therapeutic agent according to claim 36 [or 38] wherein said antibody is a recombinant antibody.

40. (Once Amended) The preventive or therapeutic agent according to claim 36 [or 39] wherein said antibody is an altered antibody.

41. (Once Amended) The preventive or therapeutic agent according to claim 36 [, 39, or 40] wherein said altered antibody is a chimeric antibody or a humanized antibody.

43. (Once Amended) The preventive or therapeutic agent according to claim 36 [or any one of claims 39-42] wherein said antibody is a modified antibody.

48. The preventive or therapeutic agent according to claim 45 [or 47] wherein said antibody is a recombinant antibody.

49. (Once Amended) The preventive or therapeutic agent according to claim 45 [or 48] wherein said antibody is an altered antibody.

50. (Once Amended) The preventive or therapeutic agent according to claim 45 [, 48, or 49] wherein said altered antibody is a chimeric antibody or a humanized antibody.

52. (Once Amended) The preventive or therapeutic agent according to claim 43 [or any one of claims 48-51] wherein said antibody is a modified antibody.

DESCRIPTION

PREVENTION AND TREATMENT OF BLOOD COAGULATION-RELATED DISEASES

FIELD OF THE INVENTION

The present invention relates to a method of generating an animal model having a persistently hypercoagulable state, preventive or therapeutic agents for diseases having a persistent hypercoagulable state, preventive or therapeutic agents for a hypercoagulable state resulting from infections, preventive or therapeutic agents for venous or arterial thrombosis, and preventive or therapeutic agents for diseases resulting from the hypertrophy of vascular media.

BACKGROUND ART

Blood coagulation is a reaction in which serine protease precursors are successively activated by activated-form proteases, which finally generate thrombin thereby leading to fibrin formation. Thrombosis arises as a consequence of an excessively enhanced blood coagulation reaction that was caused by changes in the plasma coagulation and fibrinolytic system, and in the functions of platelets, leucocytes and vascular endothelial cells associated with the progression of various disease states. The initiating factor of the blood coagulation reaction is tissue factor. In acute coronary syndromes such as acute myocardial infarction and unstable angina, the blood coagulation reaction is triggered when tissue factor occurring in abundance in the plaques formed after the progression of arterial sclerosis is exposed to the blood due to the rhexis of plaques.

In the disseminated intravascular coagulation syndrome associated with sepsis and malignant tumors, activated monocytes and macrophages express tissue factor

or tumor cells express tissue factor thereby causing enhanced blood coagulation. Once tissue factor comes into contact with the blood, the blood coagulation reaction proceeds in a very short period of time and leads to the formation of blood clots. Thus, in order to prevent thrombus formation, it is necessary to block blood coagulation reactions that may be triggered at any time or that may be constantly occurring. Therefore, an experimental model that exhibits a hypercoagulable state on a persistent basis is essential for the development of effective anti-thrombotic agents. In any of the conventionally known thrombotic models, thrombus formation is induced in a short period of time.

Thus, according to one aspect of the present invention, there is provided an experimental model in which a hypercoagulable state persists by bringing human tissue factor into contact with the blood on a persistent basis.

Blood coagulation is a reaction in which serine protease precursors are successively activated by activated-form proteases, which finally generate thrombin thereby leading to fibrin formation. Thrombosis arises as a consequence of an excessively enhanced blood coagulation reaction that was caused by changes in the plasma coagulation and fibrinolytic system, and in the functions of platelets, leucocytes and vascular endothelial cells associated with the progression of various disease states. The initiating factor of the blood coagulation reaction is tissue factor (TF).

In acute coronary syndromes such as acute myocardial infarction and unstable angina, the blood coagulation reaction is triggered when tissue factor occurring in abundance in the plaques formed after the progression of arterial sclerosis is exposed to the blood due to the rupture of plaques. In disseminated intravascular coagulation syndrome associated with sepsis and malignant tumors, activated monocytes and macrophages express TF or

tumor cells express TF thereby leading to enhanced blood coagulation and this state persists. Once TF comes into contact with the blood, the blood coagulation reaction proceeds in a very short period of time leading to the formation of blood clots. Thus, in order to prevent thrombus formation, it is necessary to block blood coagulation reactions that may be triggered at any time or that may be constantly occurring. Therefore, as an effective anti-thrombotic agent, a drug is required that can block the persistence of the hypercoagulable state that is occurring on a constant basis.

Thus, according to the second aspect of the present invention, there is provided a novel preventive or therapeutic agent for diseases having a persistent hypercoagulable state.

Severe infections are often associated with abnormal coagulation, which induces disease states such as multiple organ failure and the disseminated intravascular coagulation syndrome, and represents a factor that aggravates the prognosis of the patient. The measures employed are thus considered to be important. In severe infections, systemic infections such as sepsis and, among them, lesions in the vascular endothelial cells have been implicated as the onset mechanism of organ disorders. In sepsis, and particular in sepsis caused by gram negative bacteria, a cellular component, lipopolysaccharide (LPS), plays an important role.

LPS liberated into the blood not only activates monocytes and thereby produces tissue factor (TF) leading to a hypercoagulable state, but produces and liberates cytokines such as TNF, IL-1β and IL-8 and thereby activates neutrophils and vascular endothelial cells. The activated neutrophils adhere to the vascular endothelial cells to liberate cytotoxic substances such as active enzymes and elastases, which injure the vascular endothelial cells. In the vascular endothelial cells activated by cytokines or injured by neutrophils,

the production of TF is enhanced which further progresses the hypercoagulable state. As a result, microthrombi occur systemically, which elicits circulatory failures in the organs leading to multiple organ failure.

Thus, there is a great need for the development of preventive or therapeutic agents for blood coagulable states caused by infections.

Thus, according to the third aspect of the present invention, there is provided a novel preventive or therapeutic agent for blood coagulable states caused by infections.

As a mechanism leading to the onset of venous thrombosis, venous stasis, damages to the venous wall, and hypercoagulabilyty are thought to play an important role. In particular, invasive events such as surgery, childbirth and trauma induce physical injuries to the vascular wall and abnormalities in the coagulation and fibrinolysis system, and decubitus after surgery induces a renous stasis. Not only the resulting blood clots in the vein induce circulatory failure in the limbs but the clots themselves enter into the blood circulation and flows into the pulmonary artery leading to fatal pulmonary embolism. Hence, the prevention of venous thrombosis itself is considered to be important. Thus, there is a need for the development of agents that can effectively prevent or treat venous thrombosis.

Thus, according to the fourth aspect of the present invention, there is provided a novel preventive or therapeutic agent for the treatment of venous thrombosis.

In arterial thrombosis, blood clots occur in the blood vessel having an advanced sclerosis, and the onset of the disease in the important organs such as the brain and the heart would be fatal in most cases. In particular, acute coronary syndromes such as unstable angina and acute myocardial infarction are believed to be dangerous disease states that could easily cause sudden death. Recently it was demonstrated that the rhexis of

the arteriosclerotic plaques and the ensuing thrombus formation is an important factor in the onset mechanism of the disease.

It has also been demonstrated that tissue factor (TF), an initiating factor for thrombus formation, is being excessively expressed on the cell surface and the extracellular interstitium in the plaque, and thus it is believed that the exposure of tissue factor (TF) to the blood resulting from the rupture of plaques is a major factor for thrombus formation.

Thus, there is a great need for the development of a novel drug for preventing or treating arterial thrombosis.

Thus, according to the fifth aspect of the present invention, there is provided a novel preventive or therapeutic agent for arterial thrombosis.

Percutaneous transluminal coronary angioplasty (PTCA) occupies an important position in the treatment of coronary heart diseases. But restenosis that occurs several months after the operation hinders the effectiveness of the treating method and thus is posing a problem. As a cause of restenosis, it is becoming increasingly clear, thrombus formation during the acute phase and the subacute phase resulting from the injuries to endothelial cells is important. The contact with the blood of tissue factor (TF) expressed by the injured endothelial cells and the smooth muscles and fibroblasts in the subendothelial tissue is important for thrombus formation. The cells in the blood vessel wall grow so as to cover the resulting thrombi and thereby narrow the area of the lumen in the blood vessel. The growth of the blood vessel tissue per se and the constriction of the blood vessel diameter also contribute to the narrowing of the area of the lumen in the blood vessel, and they provide a direct factor for restenosis. Thus, there is a great need for a novel drug that can prevent or treat restenosis.

Thus, according to the sixth aspect of the present invention, there is provided a novel preventive or therapeutic agent for diseases caused by the hypertrophy of vascular media.

DISCLOSURE OF THE INVENTION

After intensive and extensive research to resolve the above first problem, the inventors of the present invention have found out that by implanting, into an experimental animal, an animal cell capable of constantly expressing human tissue factor by introducing therein the gene of a human tissue factor (TF) and thereby increasing the concentration of human tissue factor in the animal, the hypercoagulable state of said animal can be maintained for a long period of time, and thereby have completed the present invention.

Thus, according to the first aspect, the present invention provides an experimental animal having implanted therein an animal cell to which the gene encoding human tissue factor (TF) or part thereof has been inserted and which is capable of expressing said gene, said animal being a non-human animal in which a hypercoagulable state persists for a long period of time.

The part of said human tissue factor is for example a human tissue factor that lacks the intracellular region. Said animal cell is preferably a mammalian cell. Said mammalian cell is preferably a human myeloma cell. Said animal is preferably a mouse. Said hypercoagulable state is indicated by at least one of the phenomena comprising an increase in the plasma concentration of human tissue factor, a decrease in platelets, a decrease in fibrinogen, an increase in the concentration of soluble fibrin monomer complex, and an increase in the concentration of thrombin-antithrombin III complex.

The present invention also provides a method of generating the above animal, wherein an animal cell to which the gene encoding human tissue factor (TF) or part

thereof has been inserted and which is capable of expressing said gene is implanted to non-human animals and then an animal having a persistent hypercoagulable state is selected.

The present invention also provides a method of screening an anti-thrombotic agent which method comprises using the above animal.

After intensive and extensive research to resolve the above second problem, the inventors of the present invention have found out that an antibody (anti-human TF antibody, or sometimes referred to as anti-TF antibody) against human tissue factor can prevent the persistence of a hypercoagulable state.

Thus, according to the second aspect, the present invention provides a preventive or therapeutic agent for diseases having a persistent hypercoagulable state, said agent comprising an antibody against human tissue factor (human TF).

The above antibody is for example a polyclonal antibody. The above antibody is preferably a monoclonal antibody. The above antibody is preferably a recombinant antibody. The above antibody is preferably an altered antibody. The above altered antibody is preferably a chimeric antibody or a humanized antibody. The above humanized antibody is a humanized antibody of version b-b, i-b, or i-b2. The above antibody is for example an antibody modification. The above antibody modification is for example an antibody fragment Fab, $F(ab')_2$, or Fv, or a single chain Fv (scFv).

After intensive and extensive research to resolve the above third problem, the inventors of the present invention have found out that an antibody (anti-human TF antibody, or sometimes referred to as anti-TF antibody) against human tissue factor can prevent or treat a hypercoagulable state resulting from infections.

Thus, according to the third aspect, the present invention provides a preventive or therapeutic agent for

a hypercoagulable state resulting from infections, said agent comprising an antibody against human tissue factor (human TF).

The above antibody is for example a polyclonal antibody. The above antibody is preferably a monoclonal antibody. The above antibody is preferably a recombinant antibody. The above antibody is preferably an altered antibody. The above altered antibody is preferably a chimeric antibody or a humanized antibody. The above humanized antibody is a humanized antibody of version b-b, i-b, or i-b2. The above antibody is for example an antibody modification. The above modified antibody is for example an antibody fragment Fab, $F(ab')_2$, or Fv, or a single chain Fv (scFv).

After intensive and extensive research to resolve the above fourth problem, the inventors of the present invention have found out that an antibody (anti-human TF antibody, or sometimes referred to as anti-TF antibody) against human tissue factor can prevent or treat venous thrombosis.

Thus, according to the fourth aspect, the present invention provides a preventive or therapeutic agent for venous thrombosis, said agent comprising an antibody against human tissue factor (human TF).

The above antibody is for example a polyclonal antibody. The above antibody is preferably a monoclonal antibody. The above antibody is preferably a recombinant antibody. The above antibody is preferably an altered antibody. The above altered antibody is preferably a chimeric antibody or a humanized antibody. The above humanized antibody is a humanized antibody of version b-b, i-b, or i-b2. The above antibody is for example an antibody modification. The above antibody modification is for example an antibody fragment Fab, $F(ab')_2$, or Fv, or a single chain Fv (scFv).

After intensive and extensive research to resolve the above fifth problem, the inventors of the present

invention have found out that an antibody (anti-human TF antibody, or sometimes referred to as anti-TF antibody) against human tissue factor can prevent or treat arterial thrombosis.

Thus, according to the fifth aspect, the present invention provides a preventive or therapeutic agent for arterial thrombosis, said agent comprising an antibody against human tissue factor (human TF).

The above antibody is for example a polyclonal antibody. The above antibody is preferably a monoclonal antibody. The above antibody is preferably a recombinant antibody. The above antibody is preferably an altered antibody. The above altered antibody is preferably a chimeric antibody or a humanized antibody. The above humanized antibody is a humanized antibody of version b-b, i-b, or i-b2. The above antibody is for example an antibody modification. The above antibody modification is for example an antibody fragment Fab, $F(ab')_2$, or Fv, or a single chain Fv (scFv).

After intensive and extensive research to resolve the above sixth problem, the inventors of the present invention have found out that an antibody (anti-human TF antibody, or sometimes referred to as anti-TF antibody) against human tissue factor can prevent or treat diseases caused by the hypertrophy of vascular media.

Thus, according to the sixth aspect, the present invention provides a preventive or therapeutic agent for diseases caused by the hypertrophy of vascular media, said agent comprising an antibody against human tissue factor (human TF).

The above antibody is for example a polyclonal antibody. The above antibody is preferably a monoclonal antibody. The above antibody is preferably a recombinant antibody. The above antibody is preferably an altered antibody. The above altered antibody is preferably a chimeric antibody or a humanized antibody. The above humanized antibody is a humanized antibody of version b-

b, i-b, or i-b2. The above antibody is for example an antibody modification. The above antibody modification is for example an antibody fragment Fab, $F(ab')_2$, or Fv, or a single chain Fv (scFv).

BRIEF EXPLANATION OF THE DRAWINGS

Figure 1 is a graph that compares the antigen binding activity of a H chain chimeric/L chain chimeric antibody, a H chain humanized version b/L chain humanized version b antibody, a H chain humanized version i/L chain humanized version b antibody, and a H chain humanized version i/L chain humanized version b2 antibody.

Figure 2 is a graph that compares the activity of neutralizing human TF (the activity of TF to inhibit the production of Factor Xa) of a H chain chimeric/L chain chimeric antibody, a H chain humanized version b/L chain humanized version b antibody, a H chain humanized version i/L chain humanized version b antibody, and a H chain humanized version i/L chain humanized version b2 antibody.

Figure 3 is a graph that compares the activity of neutralizing human TF (the activity of TF to inhibit the production of Factor X) of a H chain chimeric/L chain chimeric antibody, a H chain humanized version b/L chain humanized version b antibody, a H chain humanized version i/L chain humanized version b antibody, and a H chain humanized version i/L chain humanized version b2 antibody.

Figure 4 is a graph that compares the activity of neutralizing human TF (the activity of TF to inhibit plasma coagulation) of a H chain chimeric/L chain chimeric antibody, a H chain humanized version b/L chain humanized version b antibody, a H chain humanized version i/L chain humanized version b antibody, and a H chain humanized version i/L chain humanized version b2 antibody.

Figure 5 is a graph showing changes in tumor volume

with time after the implantation of tumor cells in the mice implanted with the cells to which gene of human tissue factor has been introduced (dotted line) and in the mice implanted with the cells to which said gene has not been introduced (solid line).

Figure 6 is a graph showing changes in the plasma concentration of human tissue factor with time after the implantation of tumor cells in the mice implanted with the cells to which gene of human tissue factor has been introduced (dotted line) and in the mice implanted with the cells to which said gene has not been introduced (solid line).

Figure 7 is a graph showing changes in platelet counts with time after the implantation of tumor cells in the mice implanted with the cells to which gene of human tissue factor has been introduced (dotted line) and in the mice implanted with the cells to which said gene has not been introduced (solid line).

Figure 8 is a graph showing changes in the plasma concentration of fibrinogen with time after the implantation of tumor cells in the mice implanted with the cells to which gene of human tissue factor has been introduced (dotted line) and in the mice implanted with the cells to which said gene has not been introduced (solid line). The points indicate relative values, in which the concentration of fibrinogen in the control mice to which tumor cells have not been implanted (normal) is expressed as 100%.

Figure 9 is a graph showing changes in the plasma concentration of soluble fibrin monomer complex (sFMC) with time after the implantation of tumor cells in the mice implanted with the cells to which gene of human tissue factor has been introduced (dotted line) and in the mice implanted with the cells to which said gene has not been introduced (solid line).

Figure 10 is a graph showing changes in the plasma concentration of thrombin-antithrombin III complex (TAT)

with time after the implantation of tumor cells in the mice implanted with the cells to which gene of human tissue factor has been introduced (dotted line) and in the mice implanted with the cells to which said gene has not been introduced (solid line).

Figure 11 is a graph showing changes in platelet counts with time in the mice that are administered anti-human tissue factor antibody at 1 mg/kg once weekly for three weeks from day 45 after implantation of tumor cells to which gene of human tissue factor has been introduced.

Figure 12 is a graph showing the plasma concentration of soluble fibrin monomer complex (sFMC) on day 6 after the final administration of anti-human tissue factor antibody in the mice that are administered said antibody at 1 mg/kg once weekly for three weeks from day 45 after implantation of tumor cells to which gene of human tissue factor has been introduced.

Figure 13 is a graph showing the plasma concentration of thrombin-antithrombin III complex (TAT) on day 6 after the final administration of anti-human tissue factor antibody in the mice that are administered said antibody at 1 mg/kg once weekly for three weeks from day 45 after implantation of tumor cells to which gene of human tissue factor has been introduced.

Figure 14 is a graph showing changes in platelet counts with time in the mice that received the single administration of anti-human tissue factor antibody at 1 mg/kg or in the mice that received the 24-hour continuous administration of low molecular weight heparin at 601.5 IU/kg, 1900.3 IU/kg, or 6487.3 IU/kg using an osmotic pump on day 49 after implantation of tumor cells to which gene of human tissue factor has been introduced.

BEST MODE FOR CARRYING OUT THE INVENTION

The gene that encodes human tissue factor (TF) for use in the first aspect of the present invention has already been cloned, and the base sequence and the amino

acid sequence encoded thereby are also known (H. Morrissey et al., Cell, Vol. 50, p. 129-135 (1987)). The base sequence encoding the full-length human tissue factor and the corresponding amino acid sequence are set forth in SEQ ID NO: 103 and 104. According to the present invention, there may be used a gene encoding TF from which the intracellular region has been removed or a gene encoding the portion that retains the activity of initiating the blood coagulation system.

As a vector for introducing this gene into an animal cell and expressing it, any expression vector that functions in animal cells can be used, including, for example, pCOS1, pSV2-neo, pMAM-neo, and pSG5. In accordance with the present invention, a commonly used useful promoter, the human tissue factor gene, and a poly A signal, to 3'-end downstream thereof, can be functionally linked and can be expressed. As the promoter/enhancer, there can be mentioned human cytomegalovirus immediate early promoter/enhancer, viral promoters such as promoters of retrovirus, polyoma virus, adenovirus, and simian virus 40 (SV40), and promoters/enhancers derived from mammalian cells such as human elongation factor 1α (HEF1α). For expression vectors, as the replicator, there can be used those derived from SV40, polyoma virus, adenovirus and the like. Furthermore, for the expression vector can be contained as selectable markers the phosphotransferase APH (3') II or I (neo) gene, the thymidine kinase (TK) gene, the dihydrofolate reductase (dhfr) gene and the like.

As the method of introducing a gene into a cell, there can be used the electroporation method, the calcium phosphate method, the lipofection method and the like. As the cell for introducing the expression vector, any cell can be used as long as it can be grafted to an animal cell. For this purpose, various cultured cells may be used, including for example a mammalian cell such

as a cultured cell derived from human, mice, rats, hamsters, and monkeys, with tumor cells being most preferred. As specific examples of the cell, there can be used a human myeloma cell line such as KPMM2 and ARH-77, a mouse leukemia cell line such as P815, P388, and L1210.

The experimental animals for use in the present invention are mammals other than the human and are preferably small experimental animals such as mice, rats, and hamsters with the mice being most preferred.

In accordance with the second aspect of the present invention, the hypercoagulable state means a physical condition induced by human TF, and give signs as a decrease in platelet counts and fibrinogen concentration, an increase in the concentration of soluble fibrin monomer complex (SFMC) and thrombin-antithrombin III complex (TAT).

Although the antibody used in the present invention may be either polyclonal antibody or monoclonal antibody provided it has a preventive or therapeutic effect on the persistence of a hypercoagulable state due to TF, monoclonal antibody is preferably. In addition, chimeric antibody, humanized antibody or single chain Fv and so forth based on monoclonal antibody can also be used, while humanized antibody is particularly preferable.

Although the antibody used in the third aspect of the present invention may be either polyclonal antibody or monoclonal antibody provided it has a preventive or therapeutic effect on the persistence of a hypercoagulable state due to TF, monoclonal antibody is preferable. In addition, chimeric antibody, humanized antibody or single chain Fv and so forth based on monoclonal antibody can also be used, while humanized antibody is particularly preferable.

Although the antibody used in the fourth aspect of the present invention may be either polyclonal antibody or monoclonal antibody provided it has a preventive or

therapeutic effect on the persistence of a hypercoagulable state due to TF, monoclonal antibody is preferable. In addition, chimeric antibody, humanized antibody or single chain Fv and so forth based on monoclonal antibody can also be used, while humanized antibody is particularly preferable.

Although the antibody used in the fifth aspect of the present invention may be either polyclonal antibody or monoclonal antibody provided it has a preventive or therapeutic effect on the persistence of a hypercoagulable state due to TF, monoclonal antibody is preferable. In addition, chimeric antibody, humanized antibody or single chain Fv and so forth based on monoclonal antibody can also be used, while humanized antibody is particularly preferable.

Although the antibody used in the sixth aspect of the present invention may be either polyclonal antibody or monoclonal antibody provided it has a preventive or therapeutic effect on the persistence of a hypercoagulable state due to TF, monoclonal antibody is preferably. In addition, chimeric antibody, humanized antibody or single chain Fv and so forth based on monoclonal antibody can also be used, while humanized antibody is particularly preferable.

1. Anti-human TF antibody

The anti-human TF antibody used in the present invention may be of any origin, type (monoclonal or polyclonal) and form provided it has the effect of preventing or treating viral hemorrhagic fever.

The anti-human TF antibody used in the present invention can be obtained as polyclonal or monoclonal antibody using a known means. Monoclonal antibody of mammalian origin is particularly preferable as the anti-human TF antibody used in the present invention. Monoclonal antibody of mammalian origin includes that produced in hybridomas as well as that produced in a host transformed with an expression vector containing antibody

gene by genetic engineering techniques. This antibody is an antibody that inhibits the induction of thrombus by human TF by binding with human TF.

2. Antibody-Producing Hybridoma

Monoclonal antibody-producing hybridoma can basically be produced in the following manner using known technology. Namely, using human TF or a portion (fragment) of it as sensitizing antigen, this is immunized in accordance with ordinary immunization methods, the resulting immunocytes are fused with known parent cells in accordance with ordinary cell fusion methods, and those cells that produce monoclonal antibody are screened in accordance with ordinary screening methods to produce monoclonal antibody.

More specifically, monoclonal antibody should be produced in the manner described below.

To begin with, human TF used as sensitizing antigen for antibody acquisition is obtained by expressing the TF gene/amino acid sequence disclosed in J.H. Morissey, et al., Cell, Vol. 50, p. 129-135 (1987). Namely, gene sequence coding for human TF is inserted into a known expression vector to transform suitable host cells followed by purifying the target human TF protein present in the host cells or culture supernatant using a known method. This method is described in Reference Example 1 of the present specification. Moreover, the human TF used as antigen can be used by extracting and purifying from a TF-containing biological material such as human placenta according to the method described in Reference Example 2.

Next, this purified human TF protein is used as sensitizing antigen. Alternatively, soluble TF from which the membrane permeating region of the C-terminal of human TF has been removed can be produced by, for example, genetic recombination, and this can also be used as sensitizing antigen.

Although there are no particular restrictions on the

mammal that is sensitized with sensitizing antigen, it is preferable to select a mammal in consideration of compatibility with the parent cells used in cell fusion, typical examples of which include rodents such as mice, rats, hamsters, or rabbits and monkeys.

Immunization of animals with sensitizing antigen is performed in accordance with known methods. For example, as a typical immunization method, immunization is performed by injecting sensitizing antigen into the abdominal cavity or under the skin of the mammal. More specifically, sensitizing antigen is diluted to a suitable volume with phosphate-buffered saline (PBS) or physiological saline, and the resulting suspension is mixed with a suitable amount of ordinary adjuvant such as Freund's complete adjuvant as desired followed by emulsifying and administering in multiple doses to mammals every 4-21 days. In addition, a suitable carrier can also be used when immunizing with sensitizing antigen.

After immunizing the mammals in this manner and confirming that antibody has risen to the desired level in the serum, immunocytes are sampled from the mammals and applied to cell fusion. However, spleen cells are a particularly preferable example of immunocytes.

Mammalian myeloma cells are used for the other parent cells fused with the above immunocytes. Various known cell lines are used for these myeloma cells, preferable examples of which include P3 (P3x63Ag8.653) (Kearney, J.F. et al., J. Immunol. (1979) 123, 1548-1550), P3x63Ag8U.1 (Yelton, D.E. et al., Current Topics in Microbiology and Immunology (1978) 81, 1-7), NS-1 (Kohler, G. and Milstein, C., Eur. J. Immunol. (1976) 6, 511-519), MPC-11 (Margulies, D.H. et al., Cell (1976) 8, 405-415), SP2/0 (Shulman, M. et al., Nature (1978) 276, 269-270), F0 (de St. Groth, S.F. and Scheidegger, D.J., J. Immunol. Methods (1980) 35, 1-21), S194 (Trowbridge, I.S., J. Exp. Med. (1978) 148, 313-323) and R210 (Galfre,

G. et al., Nature (1979) 277, 131-133).

Cell fusion of the above immunocytes and myeloma cells can basically be carried out in compliance with known methods such as the method of Milstein, et al. (Galfre G. and Milstein, C., Methods Enzymol. (1981) 73, 3-46).

More specifically, the above cell fusion is carried out, for example, in ordinary nutrient culture media in the presence of cell fusion promoter. Examples of cell fusion promoters used include polyethylene glycol (PEG) and Sendai virus (HVJ). Moreover, an assistant such as dimethylsulfoxide can be added to further enhance fusion efficiency as desired.

The usage ratio of immunocytes and myeloma cells can be set arbitrarily. For example, the number of immunocytes is preferably 1-10 times the number of myeloma cells. Examples of culture media used in the above cell fusion include RPMI1640 culture medium, MEM culture medium and other ordinary culture media used in this type of cell culturing that is suitable for growth of the above myeloma cell lines. Moreover, serum supplement such as fetal calf serum (FCS) can also be used in combination with the above media.

Cell fusion is carried out by adequately mixing prescribed amounts of the above immunocytes and myeloma cells in the above culture media, adding PEG solution (for example, that having a molecular weight of about 1000-6000) warmed in advance to about 37°C at a concentration of usually 30-60% (w/v) and mixing to form the target fused cells (hybridoma). Subsequently, a suitable amount of culture media is sequentially added, and cell fusion agents and so forth undesirable for hybridoma growth are removed by repeated removal of supernatant by centrifugation.

The hybridoma obtained in this manner is selected by culturing in an ordinary selective culture medium such as HAT culture medium (culture medium containing

hypoxanthine, aminopterin and thymidine). Culturing in the above HAT culture medium is continued for an adequate amount of time (normally from several days to several weeks) for killing cells other than the target hybridoma cells (non-fused cells). Next, routine critical dilution is performed followed by screening for hybridoma that produces the target antibody and monocloning.

In addition, besides obtaining the above hybridoma by immunizing animals other than humans with antigen, a desired human antibody having binding activity to human TF can be obtained by sensitizing human lymphocytes to human TF in vitro, and fusing the sensitized lymphocytes with human myeloma cells such as myeloma cell line U266 having permanent mitotic ability (refer to Japanese Examined Patent Publication No. 1-59878). Moreover, human antibody to anti-human TF may also be acquired from attenuated cells by administering human TF serving as antigen to transgenic animals having all or a portion of the human antibody gene repertoire, acquiring anti-human TF antibody-producing cells and attenuating those cells (refer to International Unexamined Patent Application No. WO 94/25585, WO 93/12227, WO 92/03918, WO 94/02602, WO 96/34096 and WO 96/33735).

Hybridoma that produces monoclonal antibody obtained in this manner can be sub-cultured in ordinary culture media, and can be stored for a long period of time in liquid nitrogen.

In order to acquire monoclonal antibody from said hybridoma, said hybridoma is cultured in accordance with routine methods followed by obtaining the culture supernatant, or the hybridoma can be administered to a compatible mammal to proliferate in that mammal followed by obtaining in the form of the ascites. The former method is suitable for obtaining highly pure antibody, while the latter method is suitable for large volume production of antibody.

An example of monoclonal antibody production is

specifically described in Reference Example 2. In this example, six types of monoclonal antibodies referred to as ATR-2, 3, 4, 5, 7 and 8 are obtained. Although all of these can be used in the present invention, ATR-5 is particularly preferable.

3. Recombinant Antibody

In the present invention, recombinant antibody produced using genetic recombination technology by cloning antibody gene from hybridoma, incorporating in a suitable vector and introducing this into a host can be used as monoclonal antibody (refer to, for example, Vandamme, A.M. et al., Eur. J. Biochem. (1990) 192, 767-775).

More specifically, mRNA that codes for the variable region (V) of anti-human TF antibody is isolated from hybridoma that produces anti-human TF antibody. Isolation of mRNA is carried out by a known method such as guanidine ultracentrifugation (Chirgwin, J.M. et al., Biochemistry (1979) 18, 5294-5299) or the AGPC method (Chomczynski, P. and Sacchi, N., Anal. Biochem. (1987) 162, 156-159) to prepare total RNA, followed by preparation of the target mRNA using an mRNA Purification Kit (Pharmacia). In addition, mRNA can also be prepared directly by using the QuickPrep mRNA Purification Kit (Pharmacia).

cDNA of the antibody V region is synthesized from the resulting mRNA using reverse transcriptase. Synthesis of cDNA is carried out using the AMV Reverse Transcriptase First-strand cDNA Synthesis Kit (Seikagaku Co.). In addition, synthesis and amplification of cDNA can also be carried out by using the 5'-Ampli FINDER RACE Kit (Clontech) and the 5'-RACE method using PCR (Frohman, M.A. et al., Proc. Natl. Acad. Sci. USA (1988) 85, 8998-9002, Belyavsky, A. et al., Nucleic Acids Res. (1989) 17, 2919-2932).

The target DNA fragment is purified from the resulting PCR product and linked with vector DNA.

Moreover, a recombinant vector is produced from this, introduced into Escherichia coli and so forth, and colonies are selected to prepare the desired recombinant vector. The base sequence of the target DNA is then confirmed by a known method such as deoxyribonucleotide chain termination.

After obtaining DNA that codes for the V region of the target anti-human TF antibody, this is incorporated into an expression vector containing DNA that codes for the desired antibody constant region (C region).

In producing the anti-human TF antibody used in the present invention, an antibody gene is incorporated into an expression vector under the control of an expression control region such as an enhancer or promoter. Next, the host cells are transformed by this expression vector to express antibody.

Expression of antibody gene may be carried out either by separately incorporating DNA that codes for antibody heavy chain (H chain) or light chain (L chain) into expression vectors and then simultaneously transforming the host cells, or by incorporating DNA that codes for H chain and L chain into a single expression vector and transforming the host cells (refer to the publication of WO 94/11523).

In addition, transgenic animals can also be used in addition to the above host cells to produce recombinant antibody. For example, recombinant antibody is produced in the form of a fused gene by inserting antibody gene at an intermediate location of a gene that codes for protein characteristically produced in breast milk. A DNA fragment containing fused gene into which antibody gene has been inserted is injected into a goat embryo, and this embryo is then introduced into a female goat. The desired antibody is obtained from the mother's milk produced by the transgenic goat born from the goat that received the embryo, or its offspring. In addition, a suitable hormone may be used in the transgenic goat to

increase the amount of breast milk containing the desired antibody produced by that transgenic goat (Ebert, K.M. et al., Bio/Technology (1994) 12, 699-702).

An example of a production method of recombinant antibody is specifically described in Reference Example 3.

4. Altered Antibody

In the present invention, in addition to the above-mentioned antibodies, genetic recombinant antibody that has been artificially altered for the purpose of decreasing heterogenic antigenicity with respect to humans can also be used, examples of which include chimeric antibody and humanized antibody. These altered antibodies can be produced using known methods.

Chimeric antibody is obtained by linking DNA that codes for the antibody V region in the manner described above and DNA that codes for human antibody C region, incorporating this in an expression vector and introducing into a host to produce antibody. Chimeric antibody that is useful in the present invention can be obtained using this known method.

Humanized antibody is also referred to as reshaped human antibody. This is the result of transplanting the complementarity determining region (CDR) of antibody of a mammal other than a human, such as mouse antibody, into the complementarity determining region of human antibody, and typical genetic recombination techniques are known for this (refer to European Unexamined Patent Publication No. EP 125023 and WO 96/02576).

More specifically, a DNA sequence designed so as to link the CDR of mouse antibody with the framework region (FR) of human antibody is synthesized by PCR using as primer a plurality of oligonucleotides prepared so as to have a portion that overlaps the terminal regions of both CDR and FR (refer to the method described in the publication of WO 98/13388).

A region in which the complementarity determining

region forms a satisfactory antigen binding site is selected for the framework region of the human antibody that is linked by way of CDR. The amino acids of the framework region in the variable region of the antibody may be substituted as necessary so that the complementarity determining region of reshaped human antibody forms an appropriate antigen binding site (Sato, K. et al., Cancer Res. (1993) 53, 851-856).

The C region of human antibody is used for the C region of chimeric antibody and humanized antibody, and for example, Cγ1, Cγ2, Cγ3 and Cγ4 can be used in the H chain, while Cκ and Cλ can be used in the L chain. In addition, human antibody C region may be modified to improve the stability of the antibody or its production.

Chimeric antibody is composed of the variable region of antibody originating in a mammal other than humans and the constant region of human antibody. On the other hand, humanized antibody is composed of the complementarity determining region of an antibody originating in a mammal other than humans and the framework region and C region of human antibody. Since humanized antibody has decreased antigenicity in the human body, it is useful as an effective ingredient of the therapeutic agent of the present invention.

The production method of chimeric antibody is specifically described in Reference Example 4.

In addition, the production method of humanized antibody is specifically described in Reference Example 5. In this reference example, versions a, b, c, d, e, f, g, h, i, j, b1, d1, b3 and d3 having the amino acid sequences shown in Tables 1 and 2 were used as the humanized heavy chain (H chain) variable region (V region).

Table 1

Amino Acid Sequences of H Chain V Region

	FR1			CDR1	FR2		CDR2	
	1	2	3		4		5	6
	12345678901234567890123456789012345				67890123456789		012A3456789012345	
L39130(a)	QVQLLESGAVLARPGTSVKISCKASGFNIK				DYYMH		WVKQRPGQGLEWIG	
Z34963(b)	----------	----------	----------	-----	----------		----------	
M30885(c)	----------	----------	----------	-----	----------		----------	
M62723(d)	----------	----------	----------	-----	----------		----------	
Z80844(e)	----------	----------	----------	-----	----------		----------	
L04345(f)	----------	----------	----------	-----	----------		----------	
S78322(g)	----------	----------	----------	-----	----------		----------	
Z26827(h)	----------	----------	----------	-----	----------		----------	
U95239(i)	----------	----------	----------	-----	----------		----------	
L03147(j)	----------	----------	----------	-----	----------		----------	
P01742(b1)	----------	----------	----------	-----	--R-A-------M-		----------	
P01742(d1)	----------	----------	----------	-----	--R-A-------M-		----------	
Z80844(b3)	----------	----------	----------	-----	--R-A---------		----------	
Z80844(d3)	----------	----------	----------	-----	--R-A---------		----------	

Table 2

Amino Acid Sequences of H Chain V Region (cont. from Table 1)

	FR3			CDR3	FR4	
	7	8	9	10	11	
	6789012345678901 2ABC345678901234			56789012	34567890123	
L39130(a)	RAKLTAATSASIAYLEFSSLTNEDSAVYYCAR			DSGYAMDY	WGQGTLVTVSS	
Z34963(b)	-VTI--D--TNT--M-L---RS--T-I-----			--------	----------	
M30885(c)	-VTMLVD--KNQFS-RL--V-AA-T-------			--------	----------	
M62723(d)	-VTI--DE-T-T--M-L---RS------F---			--------	----------	
Z80844(e)	-VSI--DE-TK---M-LN--RS--T---F---			--------	----------	
L04345(f)	-VTI--DT-T-T--M-LR--RSD-T-------			--------	----------	
S78322(g)	K-T---DE-S-T--MQL---RS------S---			--------	----------	
Z26827(h)	-VTMS-DK-S-A---QWT--KAS-T-I-F---			--------	----------	
U95239(i)	-VTI--D--T-TVFM-L---RS--T-------			--------	----------	
L03147(j)	-VTF--D---NT--M-LR--RSA-T-------			--------	----------	
P01742(b1)	-VTI--D--TNT--M-L---RS--T-I-----			--------	----------	
P01742(d1)	-VTI--DE-T-T--M-L---RS------F----			--------	----------	
Z80844(b3)	-VTI--D--TNT--M-L---RS--T-I-----			--------	----------	
Z80844(d3)	-VTI--DE-T-T--M-L---RS------F---			--------	----------	

In addition, versions a, b, c, b1 and b2 having the amino acid sequences shown in Table 3 were used as the humanized light chain (L chain) V region.

Table 3

Amino Acid Sequences of L Chain V Region

	FR1	CDR1	FR2	CDR2
	1 2	3	4	5
	12345678901234567890123	45678901234	567890123456789	0123456
Z37332(a)	DIQMTQSPSSLSASVGDRVTITC	KASQDIKSFLS	WYQQKPGKAPKLLIY	YATSLAD
S68699(b)	-----------------------	-----------	---------------	-------
P01607(c)	-----------------------	-----------	---------------	-------
S65921(b1)	-----------------------	-----------	-F------S--T---	-------
X93625(b2)	-----------------------	-----------	------E----S---	-------

	FR3	CDR3	FR4
	6 7 8	9	10
	78901234567890123456789012345678	901234567	8901234567
Z37332(a)	GVPSRFSGSGSGTDFTLTISSLQPEDFATYYC	LQHGESPYT	FGGGTKVEIK
S68699(b)	--------------Y-----------------	---------	----------
P01607(c)	--------------Y----------I-----	---------	----------
S65921(b1)	--------------Y-----------------	---------	----------
X93625(b2)	--------------Y-----------------	---------	----------

As a result of evaluating antigen binding ability and TF neutralization activity by combining the above various versions of H chain V region and the various versions of L chain V region, as described in Reference Examples 6 and 7, in the case of indicating as "H chain V region version" - "L chain V region version", the combinations of "b-b", "i-b" and "i-b2" exhibited particularly high activity. Furthermore, the antigen binding ability of these humanized antibodies is shown in Fig. 1, human TF neutralization activity (TF Factor Xa production inhibitory activity) is shown in Fig. 2, human TF neutralization activity (Factor X binding inhibitory activity) is shown in Fig. 3, and human TF neutralization activity (TF plasma coagulation inhibitory activity) is shown in Fig. 4.

5. Modified Antibody Substances

The antibody used in the present invention may be an antibody fragment or modified antibody substance provided it binds to human TF and inhibits human TF activity. For example, examples of antibody fragments include single chain Fv (scFv) in which Fab, $F(ab')_2$, Fv or H chain or L chain Fv is linked with a suitable linker.

More specifically, either antibody is treated with an enzyme such as papain or pepsin to produce antibody fragments, or a gene is constructed that codes for these antibody fragments, after which a fragment is inserted into an expression vector and expressed in a suitable host (refer to, for example, Co, M.S. et al., J. Immunol. (1994) 152, 2968-2976, Better, M. & Horowitz, A.H., Methods in Enzymology (1989) 178, 476-496, Plueckthun, A. & Skerra, A., Methods in Enzymology (1989) 178, 497-515, Lamoyi, E., Methods in Enzymology (1986) 121, 652-663, Rousseaux, J. et al., Methods in Enzymology (1986) 121, 663-669, and Bird, R.E. et al., TIBTECH (1991) 9, 132-137).

scFv is obtained by linking antibody H chain V region and L chain V region. In this scFv, the H chain V region and L chain V region are linked by means of a linker, and preferably by means of a peptide linker (Huston, J.S. et al., Proc. Natl. Acad. Sci., USA (1988) 85, 5879-5883). The H chain V region and L chain V region in scFv may be of any origin described as antibody in the present specification. An arbitrary single chain peptide comprised of, for example, 12-19 amino acid residues is used for the peptide linker that links the V regions.

DNA that codes for scFv is obtained by using as template the portion of DNA coding for H chain or H chain V region and DNA coding for L chain or L chain V region of the above antibody that codes for the entire or desired amino acid sequence of those sequences, amplifying by PCR using a primer pair that defines both

of its ends, and combining and amplifying DNA that codes for a peptide linker portion and primer pair defined such that both of its ends are linked with each H chain and L chain.

In addition, once DNA that codes for scFv is produced, an expression vector that contains them and a host that is transformed by said expression vector can be obtained in accordance with routine methods, and scFv can be obtained in accordance with routine methods by using that host.

These antibody fragments can be produced from a host by acquiring the gene in the same manner as previously described and expressing that gene. The term "antibody" in the present invention includes these antibody fragments.

Anti-human TF antibody coupled with various molecules such as polyethylene glycol can also be used as modified antibody substances. These modified antibody substances are also included in the "antibody" of the present invention. These modified antibody substances can be obtained by performing chemical modification on the resulting antibody. Furthermore, antibody modification methods have already been established in this field.

6. Expression and Production of Recombinant Antibody or Altered Antibody

Antibody gene constructed in the manner previously described can be expressed and acquired by known methods. In the case of mammalian cells, antibody gene can be expressed by functionally coupling a commonly used useful promoter, antibody gene to be expressed and a poly A signal downstream from its 3'-side. An example of a promoter/enhancer is human cytomegalovirus immediate early promoter/enhancer.

In addition, other examples of promoter/enhancer that can be used to express antibody used in the present invention include virus promoter/enhancer such as

retrovirus, poliovirus, adenovirus, and simean virus 40 (SV40), as well as promoter/enhancer originating in mammalian cells such as human elongation factor 1α (HEF1α).

Gene expression can be carried out easily according to the method of Mulligan, et al. (Nature (1979) 277, 108-114) in the case of using SV40 promoter/enhancer, or according to the method of Mizushima, et al. (Nucleic Acid Res. (1990) 18, 5322) in the case of using HEF1α promoter/enhancer.

In the case of E. coli, said gene can be expressed by functionally coupling a commonly used useful promoter, signal sequence for antibody secretion and the antibody gene to be expressed. Examples of promoters include lacz promoter and araB promoter. Gene can be expressed according to the method of Ward, et al. (Nature (1989) 341, 544-546; FASEB J. (1992) 6, 2422-2427) in the case of using lacz promoter, or according to the method of Better, et al. (Science (1988) 240, 1041-1043) in the case of using araB promoter.

The pelB signal sequence (Lei, S.P. et al., J. Bacteriol. (1987) 169, 4379-4383) should be used as the signal sequence for antibody secretion in the case of producing in periplasm of E. coli. After isolating the antibody produced in periplasm, the antibody is used after suitably refolding the antibody structure.

Replication origins originating in SV40, poliovirus, adenovirus or bovine papilloma virus (BPV) and so forth can be used as replication origins. Moreover, in order to amplify the number of gene copies in host cell systems, the expression vector can contain as selection marker aminoglycoside transferase (APH) gene, thymidine kinase (TK) gene, E. coli xanthine guanine phosphoribosyl transferase (Ecogpt) gene or dihydrofolate reductase (dhfr) gene.

An arbitrary expression system, such as a eucaryotic

cell or procaryotic cell system, can be used to produce the antibody used in the present invention. Examples of eucaryotic cells include established mammalian cell systems, insect cell systems and fungal cells such as mold cells and yeast cells, while examples of procaryotic cells include bacterial cells such as E. coli cells.

The antibody used in the present invention is preferably expressed in mammalian cells such as CHO, COS, myeloma, BHK, Vero and HeLa cells.

Next, the transformed host cells are cultured in vitro or in vivo to produce the target antibody. Culturing of host cells is carried out in accordance with known methods. For example, DMEM, MEM, RPMI1640 or IMDM can be used for the culture medium, and a serum supplement such as fetal calf serum (FCS) can be used in combination with the above media.

7. Antibody Isolation and Purification

Antibody expressed and produced as described above can be isolated from cells or host animal and purified until homogeneous. Isolation and purification of antibody used in the present invention can be carried out using an affinity column. Examples of columns using a protein A column include Hyper D, POROS and Sepharose F.F. (Pharmacia). In addition, isolation and purification methods used with ordinary proteins should be used, and there are no restrictions whatsoever on these methods. For example, antibody can be isolated and purified by suitably selecting and combining, in addition to above affinity columns, a chromatography column, filter, ultrafiltration, salting out or dialysis and so forth (Antibodies: A Laboratory Manual, Ed Harlow and David Lane, Cold Spring Harbor Laboratory, 1988).

8-1. Measurement of the inhibitory effect on the persistence of a hypercoagulable state

In order to study the efficacy of prevention or treatment of the present invention for diseases having a chronic hypercoagulable state, a novel animal model is

required, and the details of the evaluation method are described in the specification of the patent application entitled "An animal model of a chronic hypercoagulable state and a method of generating the same" by the same applicant as this invention. Specific examples of the evaluation method are described as Example 1 in this specification.

The result of the experiment that used the above humanized anti-human TF antibody version "i-b2" is shown in Example 2 and Figures 11 to 13. According to this experiment, in the animal model system shown in Example 1, after the platelet count of the mice that were implanted with the tumor cell containing the human TF gene decreased to about half of that of the mice that were not implanted with the same (5 to 6 weeks after implantation), 1 mg/kg of the humanized anti-human TF antibody version "i-b2" was repeatedly administered intravenously once a week, with a result that the platelet count was maintained at a level equal to that in the mice that were not implanted with the tumor cell till the end of the experiment, i.e., three weeks after the start of the administration.

The administration of the humanized anti-human TF antibody of the present invention suppressed the increase in the concentrations of soluble fibrin monomer complex (sFMC) and thrombin-antithrombin III complex (TAT). The result confirmed that the administration of anti-human TF antibody of the present invention prevents the persistence of a hypercoagulable state and maintains a normal state.

8-2. Confirmation of the therapeutic effect on a hypercoagulable state resulting from infections

An elongation of prothrombin time, a decrease in the plasma concentration of fibrinogen, an increase in the serum concentration of fibrin degradation products, and the like can be ascribed to the hypercoagulable state. The administration of anti-human TF antibody of the

present invention suppressed the elongation of prothrombin time, the decrease in plasma concentration of fibrinogen, and the increase in the serum concentration of fibrin degradation products induced by the continuous infusion of LPS. This result demonstrates that the anti-human TF antibody of the present invention has a preventive and/or therapeutic effect on the hypercoagulable state resulting from infections.

In Example 3, this effect is described in detail.

8-3. Confirmation of the preventive and/or therapeutic effect on venous thrombosis

In Example 4, it is described in detail that the anti-human TF antibody of the present invention has a preventive and/or therapeutic effect on venous thrombosis.

8-4. Confirmation of the preventive and/or therapeutic effect on arterial thrombosis

In Example 5, it is described in detail that the anti-human TF antibody of the present invention has a preventive and/or therapeutic effect on arterial thrombosis.

8-5. Confirmation of the preventive and/or therapeutic effect on diseases resulting from the medial thickening of blood vessels

In Example 6, it is described in detail that the anti-human TF antibody of the present invention has a preventive and/or therapeutic effect on diseases resulting from the medial thickening of blood vessels.

9. Method of administration and formulation

The therapeutic agent of the present invention is used for the purpose of preventing, treating or improving diseases having a persistent hypercoagulable state, a hypercoagulable state resulting from infections, venous thrombosis, arterial thrombosis, and diseases resulting from the hypertrophy of vascular media.

Effective dosage per administration is selected from the range of 0.001 mg to 1000 mg/kg body weight.

Alternatively, the dosage of 0.01 to 100 mg/kg, preferably 0.1 to 10 mg/kg may be selected. However, the therapeutic agent containing anti-human TF antibody of the present invention is not limited to these dosages.

Preferably the method of administration is, but is not limited to, intravenous injection, intravenous drip, and the like.

The therapeutic agent of the present invention comprising anti-human TF antibody as an active ingredient may be formulated using a standard method (Remington's Pharmaceutical Science, the latest edition, Mark Publishing Company, Easton, USA), and may contain pharmaceutically acceptable carriers and/or additives.

Examples of such carriers or additives include water, a pharmaceutically acceptable organic solvent, collagen, polyvinyl alcohol, polyvinylpyrrolidone, a carboxyvinyl polymer, carboxymethylcellulose sodium, polyacrylic sodium, sodium alginate, water-soluble dextran, carboxymethyl starch sodium, pectin, methyl cellulose, ethyl cellulose, xanthan gum, gum Arabic, casein, agar, polyethylene glycol, diglycerin, glycerin, propylene glycol, Vaseline, paraffin, stearyl alcohol, stearic acid, human serum albumin (HSA), mannitol, sorbitol, lactose, a pharmaceutically acceptable surfactant and the like.

Additives used are chosen from, but are not limited to, the above or combinations thereof, as appropriate, depending on the dosage form of the present invention. For example, when used as injections, purified anti-human TF antibody may be dissolved in a solvent such as physiological saline, a buffer, and a glucose solution, to which an anti-adsorbent such as Tween 80, Tween 20, gelatin, and human serum albumin may be added. Alternatively, they may be lyophilized so as to be dissolved and reconstituted into a dosage form before use. As the excipient for lyophilization, sugar alcohols and sugars such as mannitol and glucose may be used.

EXAMPLES

The present invention will now be explained more specifically with reference to the examples.

Example 1. Generation of experimental mice

A vector in which a gene encoding human tissue factor (SEQ ID NO: 103) had been inserted into an animal expression vector pCOS1 (hTF-pCOS1) was digested with a restriction enzyme PruI and linearized, which was then introduced into a human myeloma cell line KPMM2 (FERM P-14170) by electroporation.

pCOS1 was constructed by removing the antibody gene from HEF-PMh-gγ1 (WO 92/19759) by digesting with EcoRI and SmaI and then by ligating the EcoRI-NotI-BamHI adaptor (Takara Shuzo). This was cultured in a RPMI1640 medium (containing 20% FCS hIL-6: 4 ng/ml) containing 1 mg/ml G418, and the cells that grew were confirmed to be the expression of human tissue factor using anti-human tissue factor antibody (American Diganostica) by flow cytometry. This gave a cell line KPMM2/TF226 that has introduced a human tissue factor gene therein.

The parent strain (KPMM2/parent) before the introduction of the above human tissue factor gene and the gene-introduced strain KPMM2/TF226 were cultured in a RPMI-1640 medium containing 4 ng/ml human IL-6 and 20% bovine fetal serum. The thus-grown KPMM2/TF226 cells and the parent KPMM2/parent cells, separately, were implanted subcutaneously at 1 x 10^7 cells into the flanks of SCID mice (available from CLEA Japan, male, 7-week old, mean body weight: about 22 g), and changes with time in tumor volume, platelet counts in the blood, and plasma concentrations of human tissue factor, fibrinogen, soluble fibrin monomer complex (sFMC), and thrombin-antithrombin III complex (TAT) were investigated.

As a result, as shown in Figure 5, tumor volume increased with time in all mice. As shown in Figure 6, however, the plasma concentration of human tissue factor

increased with time in the mice having implanted therein cells to which the human tissue factor gene had been introduced, but did not increase at all in the mice having implatned therein cells to which the human tissue factor gene had not been introduced. As shown in Figure 7 and Figure 8, in the mice having implanted therein cells to which the human tissue factor gene had been introduced, each of platelets and fibrinogen decreased with time, indicating that these coagulation components in the blood were consumed. In contrast, in the mice having implanted therein cells to which the human tissue factor gene had not been introduced, no decrease (consumption) in these coagulation components in the blood was noted.

As shown in Figure 9 and Figure 10, in the mice having implanted therein cells to which the human tissue factor gene had been introduced, the plasma concentration of each of soluble fibrin monomer complex (sFMC) and thrombin-antithrombin III complex (TAT) increased with time, indicating that the hypercoagulable state is persistent. In contrast, in the mice having implanted therein cells to which the human tissue factor gene had not been introduced, no increases in the above coagulation components in the blood were noted.

From the above results, it was confirmed that, in the mice having implanted therein cells to which the human tissue factor gene had been introduced, the hypercoagulable state is persistent, confirming that the animal of the present invention is useful as a model of a chronic hypercoagulable state.

Example 2.

The effect of the humanized anti-human TF antibody version "i-b2" was investigated in the model described in Example 1. Five to six weeks after the implantation of KPMM2/TF226 to SCID mice (CLEA Japan, male, 7-week old, mean body weight: about 22 g), at when platelet counts fell to about half that of the non-tumor-implanted group,

confirming the persistence of a hypercoagulable state, and thus from day 45 after the implanted 1 mg/kg of humanized anti-human TF antibody version "i-b2" was intravenously administered once a week. As a result, on day 3 after the administration of humanized anti-human TF antibody version "i-b2", platelet counts recovered to a level higher than that in the non-tumor-implanted group, and platelet counts were maintained at a level equal to that in the non-tumor-implanted group during the period from the start of the administration to week 3 when the experiment ended.

On day 6 after the third administration of humanized anti-human TF antibody version "i-b2", the plasma concentrations of soluble fibrin monomer complex (sFMC) and of thrombin-antithrombin III complex (TAT) were determined, and it was found that the administration of the antibody suppressed their increases (Figure 12 and Figure 13). These results indicated that humanized anti-human TF antibody version "i-b2" had the effect of stably maintaining coagulation at a normal level, by a once-per-week administration in the model in which a hypercoagulable state persists for a long period.

Example 3.

LPS dissolved in physiological saline was continuously injected into a vein at a done of 1 mg/kg/hr (2 ml/kg/hr) for 6 hours to cynomolgus monkeys (imported from Chuang Primates Experimental Animal Research Center, Nanning, the People's Republic of China, equal numbers of males and females, estimated age: 5-7 years old, body weight: 2.99-5.81 kg) under isoflurane anesthesia. Intravenous administration of humanized anti-human TF antibody "version i-b2" at 0.3 mg/kg (1 ml/kg) to monkeys of the humanized anti-human TF antibody-administration group, and that of a solvent (20 mM sodium acetate/150 mM NaCl, pH 6.0) to monkeys of the control group were made respectively 10 minutes before the start of continuous injection of LPS.

At the end of continuos injection of LPS, citrated blood and normal blood were drawn via the catheter mounted to the femoral artery, and prothrombin time, plasma concentration of fibrinogen and serum concentration of fibrin degradation products were determined. As shown in Table 4, LPS injection resulted in the elongation of prothrombin time, decreases in plasma concentration of fibrinogen, and increases in serum concentration of fibrin degradation products, i.e. a hypercoagulable state, but in the monkeys that had received humanized anti-human TF antibody "version i-b2", these changes were strongly suppressed. These results reveal that humanized anti-human TF antibody "version i-b2" can suppress the hypercoagulable state resulting from infections.

Table 4

Effect of humanized anti-human TF antibody on hypercoagulability by LPS injection

	Control group (n=4)		Humanized anti-human TF antibody administration group (n=4)	
	Before LPS injection	After LPS injection	Before LPS injection	After LPS injection
Prothrombin time (seconds)	11.1 ± 0.3	15.8 ± 2.3	10.9 ± 0.3	11.7 ± 0.6
Plasma concentration of fibrinogen (mg/dl)	150 ± 20	90 ± 20	170 ± 10	160 ± 20
Serum concentration of fibrin degradation (μg/ml)	0 ± 0	43 ± 14	0 ± 0	8 ± 4

* Mean ± standard error

Example 4.

In a model of venous thrombosis induced by venostasis and a venous wall injury, the effect of humanized anti-human TF antibody on the venous thrombosis was evaluated. The venostasis was created by the

ligation of the blood vessel. The venous wall injury was induced using "polidocanol (a therapeutic agent for esophageal varices, Kreussler)".

Cynomolgus monkeys with an estimated age of 3-4 years old, weighing 2.97-3.99 kg (obtained from Chuang Primate Experimental Animal Research Center, the people's Republic of China) were used in the venous thrombosis model. The monkeys were anesthetized with mixture of isoflurane and nitrous oxide to expose the bilateral jugular veins. A segment of the exposed jugular vein was completely ligated with suture around the site proximal to the heart, and was reversibly legated with suture around the site nearer to the head so as to be loosened afterward. A catheter was inserted into the segment between the both ligation of the exposed jugular vein, from the nearer site to the heart in the direction toward the head. Blood in the segment between the ligations of the exposed jugular vein was removed through the catheter and the inside was washed with physiological saline. Via the catheter, 0.5% polidocanol was injected into the segment between the ligations of the exposed blood vessel. The catheter was removed and, at the same time, the segment of the blood vessel was reversibly ligated at the immediate upper part of the catheter insertion site.

Five minutes later, the reversible ligation at the heart side was loosened to remove polidocanol. The reversible ligation at the head side was loosened to drain a smoll amount of blood, and then the segment of the exposed blood vessel was completely at the immediate upper part of the catheter insertion site. After the segment was filled with blood, the exposed jugular vein was completely ligated at the part nearer to the head. The segment between the both complete ligations was adjusted to be 1.5 cm in length. Thirty minutes later, the wet weight of the formed clots was measured. For the assessment, the sum of the wet weight of the clots in the bilateral jugular veins was used. Humanized anti-human

TF antibody "version i-b2" was intravenously administered at a dose of 0.3 mg/kg and 1.5 mg/kg 2 hours before the start of the venous thrombus formation.

The results are shown in Table 5. The administration of humanized anti-human TF antibody led to the reduction in the weight of the clots formed. Therefore, these result indicate that humanized anti-human TF antibody has a prophylactic effect on the venous thrombus formation in this model.

Table 5

Effect of humanized anti-human TF antibody in the venous thrombosis cynomolgus monkey

		Test agent not administered (n=2)	Humanized anti-human TF antibody 0.3 mg/kg i.v. (n=2)	Humanized anti-human TF antibody 1.5 mg/kg i.v. (n=2)
Sum of the wet weight of venous clots in the left and right carotid veins (mg)		20.8 21.9	1.4 1.1	0.0 0.4
	Mean	21.4	1.3	0.2

Example 5.

In a model of arterial thrombosis induced by angiostenosis and an arterial wall injury, the effect of humanized anti-human TF antibody on the arterial thrombosis was evaluated. The angiostenosis and the arterial wall injury were made by tightly ligating a blood vessel with a 20G needle pinched therein with its tip rounded and then removing the needle. This is a model that mimics angiostenosis due to arteriosclerosis and the arterial wall injury due to the plaque rupture.

Cynomolgus monkeys with an estimated age of 3-5 years old, weighing 3.55-3.99 kg (obtained from Chuang Primates Experimental Animal Research Center, the

People's Republic of China) were used in the arterial thrombosis model. The monkeys were anesthetized with ketamine hydrochloride (intramuscular administration) and butofanol (intramuscular administration) to expose the right common carotid artery. The probe of a doppler flowmeter was placed around the exposed blood vessel, and blood flow was monitored for about 5 minutes. After confirming the constant flow of the bloodstream, angiostenosis and the arterial wall injury were induced around the proximal site to the head side of the probe.

The blood flow was observed for the subsequent 15 minutes, and the time of vascular occlusion due to thrombus formation was determined. After loosening the ligation at the right common carotid artery, humanized anti-human TF antibody was administered to the monkeys of the antibody administration group. In the left common carotid artery as well, the time of vascular occlusion due to thrombus formation was determined. Humanized anti-human TF antibody "version i-b2" was intravenously administered at a dose of 0.3 mg/kg and 1.5 mg/kg one hour before the start of the thrombus formation at the left common carotid artery.

The results are shown in Table 6. The administration of humanized anti-human TF antibody led to the reduction in the time of vascular occlusion. Therefore, these results indicate that humanized anti-human TF antibody has a prophylactic effect on the arterial thrombus formation in this model.

Table 6

Effect of humanized anti-human TF antibody in the arterial thrombosis cynomolgus monkey

	Test agent not administered (n=2)	Humanized anti-human TF antibody 0.3 mg/kg i.v. (n=2)	Humanized anti-human TF antibody 1.5 mg/kg i.v. (n=2)
The time of vascular occlusion during 15-min observation after test agent administration (minutes) [left common carotid artery]	12.2	7.2	3.5
The time of vascular occlusion during 15-min observation after test agent administration (minutes) [left common carotid artery - right common carotid artery]	+0.9	-4.4	-6.2

* All are the mean of the group.

Example 6.

Cynomolgus monkeys (purchased from KEARI Inc., monkeys raised in Vietnam, the estimated age of 4-5 years) were anesthetized under 5-10 mg/kg of Ketalar, im, and 15-20 mg/kg of pentobarbital, iv, and the neck was incised to expose the carotid artery. Via the external carotid artery, a Fogarty catheter (3-5F) was inserted and the balloon was inflated to scrape the vascular intima for five times. After scraping, the catheter was extracted and the wound was sutured. One month later, the animals were euthanized and the carotid artery was removed. At this time, the contralateral carotid artery that was not balloon-injured was extracted in a similar manner.

Humanized anti-human TF antibody "version i-b2" was intravenously administered at a dose of 0.3 mg/kg over 1

minutes, 10 minutes before the vascular injury. The extracted artery was fixed in formalin, histological specimens were prepared, and stained with a HE stain and Elastica van Gieson stain, followed by image analysis to measure the area of the intima. As a result, as shown in Figure 7, humanized anti-human TF antibody "version i-b2" strongly suppressed the hypertrophy of the intima. This indicated that humanized anti-human TF antibody "version i-b2" prevents the narrowing of the area of the lumen during the remote period by suppressing the growth of the blood vessel tissue itself, suggesting that it can effectively prevent restenosis.

Table 7

	Animal No.	Non-injured blood vessel Area of media (mm^2)	Injured blood vessel Area of media (mm^2)
Control group	1	1.06	2.15 (203%)
	2	0.74	1.45 (196%)
	3	0.82	1.78 (217%)
Anti-human TF antibody	4	0.75	1.15 (153%)
	5	0.78	0.96 (123%)
	6	0.86	0.98 (114%)

(Percentage relative to the non-injured side)

Example 7.

The effect of humanized anti-human TF antibody "version i-b2" and low molecular weight heparin was investigated in the model described in Example 1. Six to seven weeks after the grafting of KPMM2/TF226 to SCID mice (CLEA Japan, male, 7 weeks old, mean body weight: about 24 g), platelet counts reduced to about half that of the non-tumor-grafted group, confirming the persistence of the hypercoagulable state. Hence, from day 49 after the grafting 1 mg/kg of humanized anti-human TF antibody version "i-b2" was intravenously administered or low molecular weight heparin at 601.5 IU/kg, 1900.3 IU/kg, and 6487.3 IU/kg was continuously administered by subcutaneously embedding an osmotic pump that permits

sustained release for 24 hours. As a result, on day 1 after the administration of humanized anti-human TF antibody version "i-b2", platelet counts recovered, and on day 3 platelet counts were higher than that in the non-tumor-grafted group, and day 3 the effect was maintained even after day 7. In contrast, in the administration group of 6487,3 IU/kg of low molecular weight heparin, a slight recovery in platelet counts was observed one and two days after the start of the continuous administration, but on day 3 the effect disappeared though there was a slight recovery in platelets counts (Figure 14).

Reference Example 1. Method of preparing soluble human TF

Soluble human TF (shTF) was prepared in the following manner.

The gene encoding the human TF penetrating region in which amino acids at position 220 and thereafter had been replaced with the FLAG peptide M2 was inserted to a mammalian cell expression vector (containing the neomycin resistant gene and the DHFR gene), and introduced into CHO cells. For the cDNA sequence of human TF, reference was made to an article by James H. Morrissey et al. (Cell (1987) 50: 129-135). The gene sequence and the amino acid sequence of this soluble human TF are shown in SEQ ID NOs: 101 and 102. After drug selection with G418, the expressed cells were selected, which were then subjected to expression amplification with methotrexate, and the shTF-expressing cells were established.

The cells were cultured in the serum-free medium CHO-S-SFMII (GIBCO) to obtain a culture supernatant containing shTF. It was diluted 2-fold with an equal volume of a 40 mM Tris-HCl buffer (pH 8.5), which was added to the Q-Sepharose Fast Flow column (100 ml, Pharmacia Biotech) equilibrated with a 20 mM Tris-HCl buffer (pH 8.5). After washing with the same buffer containing 0.1 M NaCl, the concentration of NaCl was

changed to 0.3 M, and shTF was eluted from the column. To the shTF fraction obtained, ammonium sulfate was added to a final concentration of 2.5 M, and was centrifuged (10,000 rpm, 20 minutes) to precipitate the contaminating proteins. The supernatant was added to Butyl TOYOPEARL (30 ml, TOSOH), and then was washed with a 50 mM Tris-HCl buffer (pH 6.8) containing 2.5 M ammonium sulfate. In the 50 mM Tris-HCl buffer (pH 6.8), the concentration of ammonium sulfate was linearly reduced from 2.5 M to 0 M to permit the elution of shTF. The peak fractions containing shTF were concentrated by the Centri-Prep 10 (Amicon). The concentrate was added to the TSKgel G3000SWG column (21.5 × 600 mm, TOSOH) equilibrated with a 20 mM Tris-HCl buffer (pH 7.0) containing 150 mM NaCl, and the peak fraction of shTF was collected. It was filter sterilized with a 0.22 μm membrane filter and the product was set as the soluble human TF (shTF). The concentration of the sample was calculated assuming that the molar extinction coefficient of the sample ε = 40,130 and molecular weight = 43,210.

Reference Example 2. Preparation of anti-TF monoclonal antibody

1. Purification of human TF

The purification of TF from human placenta was carried out according to the method of Ito (Ito, T. et al., J. Biol. Chem., 114: 691-696, 1993). Thus, human placenta was homogenized in Tris buffered saline (TBS, pH 7.5) containing 1.0 mM benzamidine hydrochloride, 1 mM phenylmethylsulfonyl fluoride, 1 mM diisopropylfluoro phosphate, and 0.02% sodium azide, and then the precipitate was defatted with cold acetone. The defatted powder obtained was suspended in the above buffer containing 2% Triton X-100 to solubilize TF.

The supernatant was subjected to affinity chromatography using Concanavalin A-Sepharose 4B column (Pharmacia) and anti-TF antibody-bound Sepharose 4B

column (Pharmacia), and purified TF was obtained. This was concentrated with an ultrafiltration membrane (PM-10, Amicon) and was stored as the purified sample at 4°C.

TF content in the purified sample was quantitated by Sandwich ELISA that combined a commercially available anti-TF monoclonal antibody (American Diagnostica) and polyclonal antibody (American Diagnostica) with recombinant TF as a standard.

The purity in the purified sample was confirmed by subjecting the sample to SDS-PAGE using a 4-20% density gradient polyacrylamide gel, and silver-staining the product.

2. Immunization and the preparation of hybridoma

After mixing the purified human TF (about 70 μg/ml) with an equal volume of Freund's complete adjuvant (Difco), it was immunized subcutaneously into the abdomen of 5-week old Balb/c male mice (Nippon Charles River) at 10 μg TF/mouse. On day 12, 18, and 25, TF mixed with Freund's incomplete adjuvant was subcutaneously boosted at 5 μg/mouse TF, and as a final immunization the TF solution diluted with PBS was intraperitoneally given at 5 μg/mouse on day 32.

Three days after the final immunization, the spleen cells were prepared from four mice, and were fused to the mouse myeloma cell line P3U1 at 1/5 cell count thereof by the polyethylene glycol method. The fused cells were suspended into the RPMI-1640 medium (hereinafter referred to as RPMI-medium) (Lifetech Oriental) containing 10% fetal bovine serum, which was inoculated in 400 wells per mouse (about 400 cells/well) of a 96-well plate. On day 1, 2, 3, and 5 after the fusion, half the volume of the medium was exchanged with the RPMI-medium (hereinafter referred to as HAT-medium) containing HAT (Dainippon Seiyaku) and condimed H1 (Boehringer Mannheim GmbH) to perform HAT selection of the hybridoma.

The hybridomas selected by the screening method

described below were cloned by conducting limiting dilution twice.

For the limiting dilution, 0.8 cells was inoculated per well in two 96-well plates. For the wells in which single colony was confirmed by microscopic examination, clones were selected by the following measurement of the binding activity to TF and neutralizing activity against TF. The clones obtained were acclaimed from the HAT-medium to the RPMI-medium. After the absence of reduction in antibody production ability due to acclimation was confirmed, limiting dilution was performed again for complete cloning. By the foregoing procedure, hybridomas that produce six antibodies (ATR-2, 3, 4, 5, 7, and 8) that strongly inhibit the binding of TF/Factor VIIa complex and Factor X were established.

3. Ascites formation and antibody purification

The ascites formation of the established hybridomas were carried out according to the standard method. Thus, 10^6 hybridomas that were subcultured in vitro were intraperitoneally grafted into BALB/c male mice that had previously received twice intravenous administration of mineral oil. Ascites was collected from the mice that showed a bloated abdomen 1-2 weeks after the grafting.

The purification of antibody from ascites was carried out using the ConSepLC100 system (Millipore) equipped with the Protein A column (Nippon Gaishi).

4. Cell-ELISA

Human bladder carcinoma cells J82 (Fair D. S. et al., J. Biol. Chem., 262: 11692-11698, 1987) that are known to express TF at a high level were obtained from ATCC, and subcultured and maintained in the RPMI-medium under the condition of 37°C, 5% CO_2, and 100% humidity.

Cell-ELISA plates were prepared by inoculating J82 cells to a 96-well plate at 10^5 cells/well, culturing for one day under the above condition, removing the medium and then washing twice with phosphate buffered saline (PBS), adding a 4% paraformaldehyde solution (PFA), and

allowing to stand on ice for 10 minutes for immobilization. After PFA was removed, the plate was washed with PBS, the Tris buffer (Blocking buffer) containing 1% BSA and 0.02% sodium azide was added thereto, and the plate was stored at 4°C until use.

Cell-ELISA was carried out in the following manner. Thus, the Blocking buffer was removed from the plate prepared as above, to which an anti-TF antibody solution or a hybridoma culture supernatant was added and was reacted at room temperature for 1.5 hours. After washing with PBS containing 0.05% Tween 20, alkaline phosphatase-conjugated goat anti-mouse IgG (H+L) (Zymed) was reacted for 1 hour. After washing, 1 mg/ml p-nitrophenyl phosphate disodium (Sigma) was added, and one hour later absorbance at 405/655 nm was measured to determine the amount of anti-TF antibody that bound to the J82 cells.

5. Assay system of neutralizing activity against TF with Factor Xa activity as an index

To 50 µl of Tris buffered saline (TBS: pH 7.6) containing 5 mM $CaCl_2$ and 0.1% bovine serum albumin, 10 µl of a human placenta-derived thromboplastin solution (5 mg/ml) (Thromborel S) (Boehring) and 10 µl of a Factor VIIa solution (82.5 ng/ml) (American Diagnostics) were added, and reacted at room temperature for 1 hour to permit the formation of the TF/Factor VIIa complex. After 10 µl of a predetermined concentration of a diluted anti-TF antibody solution or the hybridoma culture supernatant and 10 µl of a Factor X solution (3.245 µg/ml) (Celsus Laboratories) were added and reacted for 45 minutes, 10 µl of 0.5 M EDTA was added to stop the reaction. Fifty µl of 2 mM S-2222 solution (Daiichi Kagaku Yakuhin) was added thereto, and changes in absorbance at 405/655 nm over 30 minutes were measured and was set as the Factor X-producing activity of TF. In this method, the activity of antibody that inhibits the

binding of the TF/Factor VIIa complex and Factor X can be determined.

6. Assay system of inhibiting activity against plasma-coagulation

Fifty μl of an appropriately diluted anti-TF antibody solution was mixed with 100 μl of a commercially available normal human plasma (Kojin Bio) and reacted at 37°C for 3 minutes. Then 50 μl of human placenta-derived thromboplastin solution (1.25 mg/ml) was added thereto, and the time to coagulation of the plasma was measured using the plasma coagulation measuring instrument (CR-A: Amelung).

7. Determination of antibody isotype

For the culture supernatant of the hybridoma and the purified antibody, the mouse monoclonal antibody isotyping kit (manufactured by Amersham) was used to confirm the isotype of antibody. The result is shown below.

Table 5

Immunoglobulin isotype of anti-TF monoclonal antibody

ATR-2	IgG1, k
ATR-3	IgG1, k
ATR-4	IgG1, k
ATR-5	IgG1, k
ATR-7	IgG2a, k
ATR-8	IgG2a, k

Reference Example 3. Cloning of DNA encoding the V region of a mouse monoclonal antibody against human TF

(1) Preparation of mRNA

mRNA was prepared from hybridoma ATR-5 (IgG1κ) obtained in Reference Example 2 using the QuickPrep mRNA Purification Kit (Pharmacia Biotech). Each hybridoma cell was completely homogenized in the extraction buffer according to instructions attached to the kit, and then

mRNA was purified by the oligo (dT)-cellulose spun column, followed by ethanol precipitation. The mRNA precipitate was dissolved in the elution buffer.

(2) Preparation and amplification of cDNA of the gene encoding a mouse antibody V region

(i) Cloning of H chain V region cDNA

The cloning of the gene encoding the H chain V region of a mouse monoclonal antibody against human TF was carried out using the 5'-RACE method (Frohman, M.A. et al., Proc. Natl. Acad. Sci. USA 85: 8998-9002, 1988; Belyavsky, A. et al., Nucleic Acids Res. 17: 2919-2932, 1989). For the 5'-RACE method, the Marathon cDNA Amplification Kit (CLONTECH) was used and the procedure carried out according to the instructions attached to the kit.

Using about 1 μg of mRNA prepared as above as a template, the cDNA synthesis primer attached to the kit was added, which was reacted with a reverse transcriptase at 42°C for 60 minutes to effect reverse transcription to cDNA. This was reacted with DNA polymerase I, DNA ligase, and RNaseH at 16°C for 1.5 hour, and with T4 DNA polymerase at 16°C for 45 minutes thereby to synthesize a double stranded cDNA. The double stranded cDNA was extracted with phenol and chloroform, and recovered by ethanol precipitation.

By overnight reaction with T4 DNA ligase at 16°C, a cDNA adapter was ligated to both ends of the double stranded cDNA. The reaction mixture was diluted 50-fold with a 10 mM Tricine-KOH (pH 8.5) containing 0.1 mM EDTA. Using this as a template, the gene encoding the H chain V region was amplified by PCR. The adapter primer 1 attached to the kit was used for the 5'-end primer, and for the 3'-end primer the MHC-G1 primer (SEQ ID NO: 1) (S. T. Jones, et al., Biotechnology, 9: 88-89, 1991) were used.

PCR solutions for the ATR-5 antibody H chain V

region contained, in 100 µl, 120 mM Tris-HCl (pH 8.0), 10 mM KCl, 6 mM $(NH_4)_2SO_4$, 0.1% Triton X-100, 0.001% BSA, 0.2 mM dNTPs (dATP, dGTP, dCTP, dTTP), 1 mM $MgCl_2$, 2.5 units of KOD DNA polymerase (Toyo Boseki), 30-50 pmole of adapter primer 1, as well as MHC-G1 primer, and 1-5 µl of a reaction mixture of cDNA to which the cDNA adapter was ligated.

All PCRs were carried out using the DNA Thermal Cycler 480 (Perkin-Elmer), and the PCR was performed for thirty cycles at a temperature cycle of 94°C for 30 seconds, 55°C for 30 seconds, and 74°C for 1 minute.

(ii) Cloning of L chain V region cDNA

The cloning of the gene encoding the L chain V region of a mouse monoclonal antibody against human TF was carried out using the 5'-RACE method (Frohman, M.A. et al., Proc. Natl. Acad. Sci. USA 85: 8998-9002, 1988; Belyavsky, A. et al., Nucleic Acids Res. 17: 2919-2932, 1989). For the 5'-RACE method, the Marathon cDNA Amplification Kit (CLONTECH) was used and carried out according to the instructions attached to the kit. Using about 1 µg of mRNA prepared as above as a template, the cDNA synthesis primer was added, which was reacted with a reverse transcriptase at 42°C for 60 minutes to effect reverse transcription to cDNA.

This was reacted with DNA polymerase I, DNA ligase, and RNaseH at 16°C for 1.5 hour, and with T4 DNA polymerase at 16°C for 45 minutes thereby to synthesize a double stranded cDNA. The double stranded cDNA was extracted with phenol and chloroform, and recovered by ethanol precipitation. By overnight reaction with T4 DNA ligase at 16°C, a cDNA adapter was ligated to both ends of the double stranded cDNA. The reaction mixture was diluted 50-fold with a 10 mM Tricine-KOH (pH 8.5) containing 0.1 mM EDTA. Using this as a template, the gene encoding the L chain V region was amplified by PCR. The adapter primer 1 was used for the 5'-end primer, and

for the 3'-end primer the MKC primer (SEQ ID NO: 2) (S. T. Jones, et al., Biotechnology, 9: 88-89, 1991) was used.

PCR solutions contained, in 100 µl, 120 mM Tris-HCl (pH 8.0), 10 mM KCl, 6 mM $(NH_4)_2SO_4$, 0.1% Triton X-100, 0.001% BSA, 0.2 mM dNTPs (dATP, dGTP, dCTP, dTTP), 1 mM $MgCl_2$, 2.5 units of KOD DNA polymerase (Toyo Boseki), 30-50 pmole of adapter primer 1, as well as MKC primer, and 1 µl of a reaction mixture of cDNA to which the cDNA adapter was ligated.

All PCRs were carried out using the DNA Thermal Cycler 480 (Perkin-Elmer), and the PCR was performed for thirty cycles at a temperature cycle of 94°C for 30 seconds, 55°C for 30 seconds, and 74°C for 1 minute.

(3) Purification and fragmentation of PCR products

The above PCR reaction mixture was extracted with phenol and chloroform, and the amplified DNA fragments were recovered by ethanol precipitation. DNA fragments were digested with the restriction enzyme XmaI (New England Biolabs) at 37°C for 1 hour. The XmaI-digestion mixture was separated by agarose gel electrophoresis using 2%-3% NuSieve GTG agarose (FMC BioProducts), and the agarose strips containing about 500 bp long DNA fragments as the H chain V region and about 500 bp Long DNA fragments as the L chain V region were excised. The agarose strips were extracted with phenol and chloroform, DNA fragments were precipitated with ethanol, which were then dissolved in 10 µl of 10 mM Tris-HCl (pH 8.0) containing 1 mM EDTA (hereinafter referred to as TE).

The XmaI-digested DNA fragments prepared as above containing a genes encoding a mouse H chain V region and L chain V region and the pUC19 plasmid vector prepared by digesting with XmaI were ligated using the DNA ligation kit ver.2 (Takara Shuzo) by reacting at 16°C for 1 hour according to the instructions attached to the kit.

The ligation mixture was added to 100 µl of E. coli

JM109 competent cells (Nippongene) and was incubated for 30 minutes on ice and for 1 minute at 42°C.

Then, 300 μl of the Hi-Competence Broth (Nippongene) was added thereto, incubated at 37°C for 1 hour. Then, Escherichia coli was plated on a LB agar medium (Molecular Cloning: A Laboratory Manual, Sambrook, et al., Cold Spring Harbor Laboratory Press, 1989) containing 100 μg/ml ampicillin (hereinafter referred to as LBA agar medium), and incubated overnight at 37°C to obtain an E. coli transformant.

The transformant was cultured overnight in 3 ml or 4 ml of a LB medium containing 50 μg/ml ampicillin (hereinafter referred to as LBA medium) at 37°C, and from the cell fractions, plasmid DNA was prepared using the QIAprep Spin Plasmid Kit (QIAGEN), and then the nucleotide sequence was determined.

(4) Determination of the nucleotide sequence of the gene encoding a mouse antibody V region

The nucleotide sequence of the cDNA coding region in the above plasmid was determined using the Dye Terminator Cycle Sequencing FS Ready Reaction Kit (Perkin-Elmer) by the DNA Sequencer 373A (Perkin-Elmer). As the sequencing primer, M13 Primer M4 (Takara Shuzo) (SEQ ID NO: 3) and M13 Primer RV (Takara Shuzo) (SEQ ID NO: 4) were used, and the sequence was determined by confirming the nucleotide sequence in both directions.

Thus obtained plasmids containing the gene encoding the mouse H chain V region derived from the hybridoma ATR-5 was designated as ATR-5Hv/pUC19, and the thus obtained plasmids containing the gene encoding a mouse L chain V region derived from the hybridoma ATR-5 was designated as ATR-5Lv/pUC19. The nucleotide sequences of the genes encoding the H chain V region of each mouse antibody contained in the plasmid ATR-5Hv/pUC19 (including the corresponding amino acid sequences) is shown in SEQ ID NO: 5 and 99, respectively, and the

nucleotide sequences of the genes encoding the L chain V region of each mouse antibody contained in the plasmid ATR-5Lv/pUC19 (including the corresponding amino acid sequences) is shown in SEQ ID NO: 6 and 100, respectively.

Reference Example 4. Construction of chimeric antibody

A chimeric ATR-5 antibody was generated in which the mouse ATR-5 antibody V region was ligated to the human antibody C region. A chimeric antibody expression vector was constructed by ligating the gene encoding the ATR-5 antibody V region to an expression vector encoding the human antibody C region.

(1) Construction of a chimeric antibody H chain V region

The ATR-5 antibody H chain V region was modified by the PCR method in order to ligate it to an expression vector encoding the human antibody H chain C region. The 5'-end primer ch5HS (SEQ ID NO: 7) was designed so as to hybridize the 5'-end of DNA encoding the V region and to have the Kozak consensus sequence (Kozak, M. et al., J. Mol. Biol. 196: 947-950, 1987) and a recognition sequence of the restriction enzyme SalI. The 3'-end primer ch5HA (SEQ ID NO: 8) was designed so as to hybridize 3'-end of DNA encoding the J region and to have a recognition sequence of the restriction enzyme NheI.

The PCR solutions contained, in 100 μl, 120 mM Tris-HCl (pH 8.0), 10 mM KCl, 6 mM $(NH_4)_2SO_4$, 0.1% Triton X-100, 0.001% BSA, 0.2 mM dNTPs (dATP, dGTP, dCTP, dTTP), 1 mM $MgCl_2$, 2.5 units of KOD DNA polymerase (Toyo Boseki), 50 pmole of the ch5HS primer and the ch5HA primer, as well as 1 μl of the plasmid ATR5Hv/pUC19 as a template DNA. For PCR, the DNA Thermal Cycler 480 (Perkin-Elmer) was used, and the PCR was performed for thirty cycles at a temperature cycle of 94°C for 30 seconds, 55°C for 30 seconds, and 74°C for 1 minute.

The PCR reaction mixture was extracted with phenol and chloroform, and the amplified DNA fragments were recovered by ethanol precipitation. The DNA fragments were digested with the restriction enzyme NheI (Takara Shuzo) at 37°C for 1 hour, and then with the restriction enzyme SalI (Takara Shuzo) at 37°C for 1 hour. The digestion mixture was separated by agarose gel electrophoresis using a 3% NuSieve GTG agarose (FMC BioProducts), and the agarose strips containing about 450 bp long DNA fragments were excised. The agarose strips were extracted with phenol and chloroform, and the DNA fragments were precipitated with ethanol, which were then dissolved in 20 μl of TE.

As the cloning vector, an altered promoter vector (hereinafter referred to as CVIDEC) was used in which the recognition sequences of the restriction enzymes NheI, SalI, and SplI, BglII were introduced. The gene fragment prepared as above encoding the mouse H chain V region and the CVIDEC vector prepared by digesting with NheI and SalI were ligated using the DNA ligation kit ver.2 (Takara Shuzo) by reacting at 16°C for 1 hour according to the instructions attached to the kit.

The ligation mixture was added to 100 μl of E. coli JM109 competent cells (Nippongene) and was incubated for 30 minutes on ice and for 1 minute at 42°C. Then, 300 μl of the Hi-Competence Broth (Nippongene) was added thereto, incubated at 37°C for 1 hour, and then the E. coli was plated on the LBA agar medium and incubated overnight at 37°C to obtain an E. coli transformant. The transformant was cultured overnight at 37°C in 3 ml of the LBA medium, and from the cell fractions, plasmid DNA was prepared using the QIAprep Spin Plasmid Kit (QIAGEN).

The nucleotide sequence of the cDNA coding region in the plasmid was determined using the Dye Terminator Cycle Sequencing FS Ready Reaction Kit (Perkin-Elmer) by the DNA Sequencer 373A (Perkin-Elmer). As the sequencing

primer, M13 Primer M4 (Takara Shuzo) (SEQ ID NO: 3) and M13 Primer RV (Takara Shuzo) (SEQ ID NO: 4) were used, and the sequence was determined by confirming the nucleotide sequence in both directions. The plasmid that contains the gene encoding the ATR-5 antibody H chain V region, a SalI recognition sequence and the Kozak consensus sequence at the 5'-end, and a NheI recognition sequence at the 3'-end was designated as chATR5Hv/CVIDEC.

(2) Construction of a chimeric antibody L chain V region

The ATR-5 antibody L chain V region was modified by the PCR method in order to ligate it to an expression vector encoding the human antibody L chain C region. The 5'-end primer ch5LS (SEQ ID NO: 9) was designed so as to hybridize to the 5'-end of the DNA encoding the V region and to have the Kozak consensus sequence (Kozak, M. et al., J. Mol. Biol. 196: 947-950, 1987) and a recognition sequence of the restriction enzyme BglII. The 3'-end primer ch5LA (SEQ ID NO: 10) was designed so as to hybridize to the 3'-end of the DNA encoding the J region and to have a recognition sequence of the restriction enzyme SplI.

The PCR solutions contained, in 100 µl, 120 mM Tris-HCl (pH 8.0), 10 mM KCl, 6 mM $(NH_4)_2SO_4$, 0.1% Triton X-100, 0.001% BSA, 0.2 mM dNTPs (dATP, dGTP, dCTP, dTTP), 1 mM $MgCl_2$, 2.5 units of KOD DNA polymerase (Toyo Boseki), 50 pmole of the ch5LS primer and the ch5LA primer, as well as 1 µl of the plasmid ATR5Lv/pUC19 as a template DNA. For PCR the DNA Thermal Cycler 480 (Perkin-Elmer) was used, and the PCR was performed for thirty cycles at a temperature cycle of 94°C for 30 seconds, 55°C for 30 seconds, and 74°C for 1 minute.

The PCR reaction mixture was extracted with phenol and chloroform, and the amplified DNA fragments were recovered by ethanol precipitation. The DNA fragments were digested with the restriction enzyme SplI (Takara

Shuzo) at 37°C for 1 hour, and then with the restriction enzyme BglII (Takara Shuzo) at 37°C for 1 hour. The digestion mixture was separated by agarose gel electrophoresis using a 3% NuSieve GTG agarose (FMC BioProducts), and the agarose strips containing about 400 bp long DNA fragments were excised. The agarose strips were extracted with phenol and chloroform, the DNA fragments were precipitated with ethanol, which were then dissolved in 20 μl of TE.

The gene fragment prepared as above encoding the mouse L chain V region and the CVIDEC vector prepared by digesting with SplI and BglII were ligated using the DNA ligation kit ver.2 (Takara Shuzo) by reacting at 16°C for 1 hour according to the instructions attached to the kit.

The ligation mixture was added to 100 μl of E. coli JM109 competent cells (Nippongene) and was incubated for 30 minutes on ice and for 1 minute at 42°C. Then, 300 μl of the Hi-Competence Broth (Nippongene) was added thereto, incubated at 37°C for 1 hour, and then the E. coli was plated on a 100 μg/ml LBA agar medium and incubated overnight at 37°C to obtain an E. coli transformant. The transformant was cultured overnight at 37°C in 3 ml of the LBA medium, and from the cell fractions, plasmid DNA was prepared using the QIAprep Spin Plasmid Kit (QIAGEN).

The nucleotide sequence of the cDNA coding region in the plasmid was determined using the Dye Terminator Cycle Sequencing FS Ready Reaction Kit (Perkin-Elmer) by the DNA Sequencer 373A (Perkin-Elmer). As the sequencing primer, M13 Primer M4 (Takara Shuzo) and M13 Primer RV (Takara Shuzo) were used, and the sequence was determined by confirming the nucleotide sequence in both directions. The plasmid that contains the gene encoding the ATR-5 antibody L chain V region and that has a BglII recognition sequence and the Kozak consensus sequence at the 5'-end and a SplI recognition sequence at the 3'-end

was designated as chATR5Lv/CVIDEC.

(3) Construction of a chimeric antibody expression vector

A chimeric antibody expression vector was constructed using an antibody expression vector introduced from IDEC Pharmaceuticals. As the vector, the IgG1-type antibody expression vector H5KG1(V) and the IgG4-type antibody expression vector N5KG4P were used. The chimeric ATR-5 antibody expression vector was generated by ligating a gene encoding the H chain V region of ATR-5 to the SalI-NheI site located immediately before the human antibody H chain C region of the expression vector N5KG1(V) or N5KG4P and ligating a gene encoding the L chain V region of ATR-5 to the BglII-SplI site located immediately before the human antibody L chain C region of the expression vector N5KG1(V) or N5KG4P.

(i) Introduction of H chain V region

The plasmid chATR5Hv/CVIDEC was digested with the restriction enzyme NheI (Takara Shuzo) at 37°C for 3 hours, and with the restriction enzyme SalI (Takara Shuzo) at 37°C for 3 hours. The digestion mixture was separated by agarose gel electrophoresis using 1.5% NuSieve GTG agarose (FMC BioProducts), and the agarose strips containing about 450 bp long DNA fragments were excised. The agarose strips were extracted with phenol and chloroform, and the DNA fragments were precipitated with ethanol, which were then dissolved in 20 μl of TE.

The expression vector N5KG1(V) and N5KG4P were digested with the restriction enzyme NheI (Takara Shuzo) at 37°C for 3 hours, and with the restriction enzyme SalI (Takara Shuzo) at 37°C for 3 hours. The digestion mixture was separated by agarose gel electrophoresis using 1.5% NuSieve GTG agarose (FMC BioProducts), and the agarose strips containing about 9000 bp long DNA fragments were excised. The agarose strips were extracted with phenol and chloroform, and the DNA

fragments were precipitated with ethanol, which were then dissolved in 20 µl of TE.

The SalI-NheI DNA fragment prepared as above containing the gene encoding the H chain V region and N5KG1(V) or N5KG4P digested with SalI and NheI were ligated using the DNA ligation kit ver.2 (Takara Shuzo) by reacting at 16°C for 1 hour according to the attached instructions.

The ligation mixture was added to 100 µl of E. coli JM109 competent cells (Nippongene) and was incubated for 30 minutes on ice and for 1 minute at 42°C. Then, 300 µl of the Hi-Competence Broth (Nippongene) was added thereto, incubated at 37°C for 1 hour, and then the E. coli was plated on a 100 µg/ml LBA agar medium and incubated overnight at 37°C to obtain an E. coli transformant. The transformant was cultured overnight at 37°C in 3 ml of the LBA medium, and from the cell fractions, plasmid DNA was prepared using the QIAprep Spin Plasmid Kit (QIAGEN). These plasmids containing the genes encoding the chimeric ATR-5 antibody H chain were designated as chATR5Hv/N5KG1(V) and chATR5Hv/N5KG4P, respectively.

(ii) Introduction of the L chain V region

The plasmid chATR5Lv/CVIDEC was digested with the restriction enzymes BglII (Takara Shuzo) and SplI (Takara Shuzo) at 37°C for 1.5 hour. The digestion mixture was separated by agarose gel electrophoresis using 1.5% NuSieve GTG agarose (FMC BioProducts), and the agarose strips containing about 400 bp long DNA fragments were excised. The agarose strips were extracted with phenol and chloroform, and the DNA fragments were precipitated with ethanol, which were then dissolved in 20 µl of TE.

The plasmids chATR5Hv/N5KG1(V) and chATR5Hv/N5KG4P were digested with the restriction enzymes BglII (Takara Shuzo) and SplI (Takara Shuzo) at 37°C for 1.5 hour. The

digestion mixture was separated by agarose gel electrophoresis using 1.5% NuSieve GTG agarose (FMC BioProducts), and the agarose strips containing about 9400 bp long DNA fragments were excised. The agarose strips were extracted with phenol and chloroform, DNA fragments were precipitated with ethanol, which were then dissolved in 20 μl of TE.

The SplI-BglII DNA fragment prepared as above containing the gene encoding the L chain V region and chATR5Hv/N5KG1(V) or chATR5Hv/N5KG4P digested with SplI and BglII were ligated using the DNA ligation kit ver.2 (Takara Shuzo) by reacting at 16°C for 1 hour according to the attached instructions.

The ligation mixture was added to 100 μl of E. coli JM109 competent cells (Nippongene) and was incubated for 30 minutes on ice and for 1 minute at 42°C. Then, 300 μl of the Hi-Competence Broth (Nippongene) was added thereto, incubated at 37°C for 1 hour, and then the E. coli was plated on a 100 μg/ml LBA agar medium and incubated overnight at 37°C to obtain an E. coli transformant. The transformant was cultured overnight at 37°C in 1 l of the 2xYT medium containing 50 μg/ml ampicillin, and from the cell fractions, plasmid DNA was prepared using the Plasmid Maxi Kit (QIAGEN). These plasmids containing the gene encoding the chimeric ATR-5 antibody were designated as chATR5/N5KG1(V) and chATR5/N5KG4P, respectively.

(4) Transfection into COS-7 cells

In order to evaluate the activity of binding to the antigen and the neutralizing activity of chimeric antibody, the above expression plasmid was transfected to COS-7 cells and the antibody was transient expressed.

The plasmid chATR5/N5KG1(V) or chATR5/N5KG4P was transduced into COS-7 cells by electroporation using the Gene Pulser instrument (Bio Rad). Fifty μg of the

plasmid was added to 0.78 ml of the COS-7 cells suspended in the Dulbecco PBS (-) (hereinafter referred to as PBS) at a cell concentration of 1×10^7 cells/ml, which was subjected to pulses of 1,500 V and 25 μF capacity.

After 10 minutes of the recovery period at room temperature, the electroporated cells were suspended in a DMEM medium containing 5% Ultra low IgG fetal bovine serum (GIBCO), and cultured using a 10 cm culture dish in a 5% CO_2 incubator. After culturing for 24 hours, the culture supernatant was aspirated off, and then a serum-free medium HBCHO (Irvine Scientific) was added. After further culturing for 72 hours, the culture supernatant was collected and centrifuged to remove cell debris.

(5) Purification of antibody

From the culture supernatant of the COS-7 cells, chimeric antibody was purified using the rProtein A Sepharose Fast Flow (Pharmacia Biotech) as follows.

One ml of rProtein A Sepharose Fast Flow was filled into a column and the column was equilibrated by 10 volumes of TBS. The culture supernatant of COS-7 cells was applied to the equilibrated column, which was then washed with 10 volumes of TBS.

The adsorbed antibody fraction was then eluted by 13.5 ml of 2.5 mM HCl (pH 3.0), and the eluate was immediately neutralized by adding 1.5 ml of 1 M Tris-HCl (pH 8.0).

By performing ultrafiltration twice for the purified antibody fraction using the Centriprep 100 (Amicon), the solvent was replaced to 50 mM Tris-HCl (pH 7.6) containing 150 mM NaCl (hereinafter referred to as TBS), and was finally concentrated to about 1.5 ml.

(6) Establishment of a stably-producing CHO cell line

In order to establish a cell line that stably produces chimeric antibody, the above expression plasmid was introduced into CHO cells (DG44) acclimated to the

CHO-S-SFMII serum-free medium (GIBCO).

The plasmid chATR5/N5KG1(V) or chATR5/N5KG4P was cleaved with the restriction enzyme SspI (Takara Shuzo) to linearize DNA, and after extraction with phenol and chloroform, DNA was recovered by ethanol precipitation. The linearized plasmid was transduced into the DG44 cells by electroporation using the Gene Pulser instrument (Bio Rad). Ten μg of the plasmid was added to 0.78 ml of DG44 cells suspended in PBS at a cell concentration of 1×10^7 cells/ml, which was subjected to pulses of 1,500 V and 25 μF capacity.

After 10 minutes of the recovery period at room temperature, the electroporated cells were suspended in a CHO-S-SFMII medium (GIBCO) containing hypoxanthine/thymidine (GIBCO), and cultured using two 96-well plates (Falcon) in a 5% CO_2 incubator. On the day after the start of culturing, the medium was changed to a selection medium containing the CHO-S-SFMII medium (GIBCO) containing hypoxanthine/thymidine (GIBCO) and 500 μg/ml GENETICIN (G418Sulfate, GIBCO) to select cells into which the antibody gene had been introduced. After changing the selection medium, the cells were examined under a microscope about two weeks later. After a favorable cell growth was observed, the amount of antibody produced was measured by the ELISA described below for determining antibody concentration, and cells having a high production yield of antibody were selected.

Reference Example 5. Construction of humanized antibody

(1) Construction of humanized antibody H chain

(i) Construction of the humanized H chain version "a"

Humanized ATR-5 antibody H chain was generated using CDR-grafting by the PCR method. In order to generate the humanized antibody H chain version "a" having the FRs derived from human antibody L39130 (DDBJ, Gao L. et al.,

unpublished, 1995), seven PCR primers were used. The CDR-grafting primers hR5Hv1S (SEQ ID NO: 11), hR5Hv2S (SEQ ID NO: 12), and hR5Hv4S (SEQ ID NO: 13) have a sense DNA sequence, and the CDR grafting primers hR5Hv3A (SEQ ID NO: 14) and hR5Hv5A (SEQ ID NO: 15) have an antisense DNA sequence, each primer having a 18-35 bp complementary sequence on both ends thereof.

hR5Hv1S was designed to have the Kozak consensus sequence (Kozak, M. et al., J. Mol. Biol. 196: 947-950, 1987) and a SalI recognition site, and hR5Hv5A was designed to have a NheI recognition site. The exogenous primer hR5HvPrS (SEQ ID NO: 16) has a homology with the CDR-grafting primer hR5Hv1S, and hR5HvPrA (SEQ ID NO: 17) has a homology with the CDR-grafting primer hR5Hv5A.

The CDR-grafting primers hR5Hv1S, hR5Hv2S, hR5Hv3A, hR5Hv4S, and hR5Hv5A, and exogenous primers hR5HvPrS and hR5HvPrA were synthesized and purified by Pharmacia Biotech.

PCR was performed using the KOD DNA polymerase (Toyo Boseki) and using the attached buffer under the condition of containing 120 mM Tris-HCl (pH 8.0), 10 mM KCl, 6 mM $(NH_4)_2SO_4$, 0.1% Triton X100, 0.001% BSA, 0.2 mM dNTPs (dATP, dGTP, dCTP, dTTP), 1 mM $MgCl_2$, 2.5 units of KOD DNA polymerase (Toyo Boseki), and 5 pmole each of the CDR-grafting primers hR5Hv1S, hR5Hv2S, hR5Hv3A, hR5Hv4S, and hR5Hv5A in 98 µl, for 5 cycles at a temperature cycle of 94°C for 30 seconds, 50°C for 1 minute, and 72°C for 1 minute. After further addition of 100 pmole of exogenous primers hR5HvPrS and hR5HvPrA, PCR was performed for 25 cycles in a system of 100 µl with the same temperature cycle. DNA fragments amplified by the PCR method were separated by agarose gel electrophoresis using a 2% NuSieve GTG agarose (FMC BioProducts).

The agarose strips containing about 430 bp long DNA fragments were excised, to which 3 volumes (ml/g) of TE was added, and then were extracted with phenol,

phenol/chloroform, and chloroform to purify the DNA fragments. After precipitating the purified DNA with ethanol, one third the volume thereof was dissolved in 17 μl of water. The PCR reaction mixture obtained was digested with NheI and SalI, and was ligated to the plasmid vector CVIDEC prepared by digesting with NheI and SalI, using the DNA ligation kit ver.2 (Takara Shuzo) according to the instructions attached to the kit.

The ligation mixture was added to 100 μl of E. coli JM109 competent cells (Nippongene) and was incubated for 30 minutes on ice and for 1 minute at 42°C. Then, 300 μl of the Hi-Competence Broth (Nippongene) was added thereto, incubated at 37°C for 1 hour, and then the E. coli was plated on the LBA agar medium and incubated overnight at 37°C to obtain an E. coli transformant. The transformant was cultured overnight at 37°C in 3 ml of the LBA medium, and from the cell fractions, plasmid DNA was prepared using the QIAprep Spin Plasmid Kit (QIAGEN).

The nucleotide sequence of the cDNA coding region in the plasmid was determined using the Dye Terminator Cycle Sequencing FS Ready Reaction Kit (Perkin-Elmer) by the DNA Sequencer 373A (Perkin-Elmer). As the sequencing primer, M13 Primer M4 (Takara Shuzo) and M13 Primer RV (Takara Shuzo) were used, and the sequence was determined by confirming the nucleotide sequence in both directions.

Since mutation and/or deletion were observed before or after the EcoT221 recognition site, each of fragments having the correct sequence was ligated and then subcloned again to CVIDEC to determine the nucleotide sequence. The plasmid having the correct sequence was designated as hATR5Hva/CVIDEC. The nucleotide sequence and the corresponding amino acid sequence of the humanized H chain version "a" contained in the plasmid hATR5Hva/CVIDEC are shown in SEQ ID NO: 18. The amino acid sequence of version "a" is also shown in SEQ ID NO: 19.

(ii) Construction of humanized H chain versions "b" and "c"

Versions "b" and "c" were generated by replacing the FR3 of version "a" with the FR3 derived from another human antibody using the FR-shuffling method. In order to replace the FR3 in version "b" with one derived from human antibody Z34963 (DDBJ, Borretzen M. et al., Proc. Natl. Acad. Sci. USA, 91: 12917-12921, 1994), the four DNA primers encoding the FR3 were generated. The FR-shuffling primers F3RFFS (SEQ ID NO: 20) and F3RFBS (SEQ ID NO: 21) have a sense DNA sequence and F3RFFA (SEQ ID NO: 22) and F3RFBA (SEQ ID NO: 23) have an antisense DNA sequence.

F3RFFS and F3RFFA have a sequence complementary to each other, and have BalI and XhoI recognition sequences on both ends. In order to replace the FR3 in version "c" with one derived from human antibody P01825 (SWISS-PROT, Poljak RJ. et al., Biochemistry, 16: 3412-3420, 1977), four DNA primers encoding the FR3 were generated. The FR-shuffling primers F3NMFS (SEQ ID NO: 24) and F3NMBS (SEQ ID NO: 25) have a sense DNA sequence and F3NMFA (SEQ ID NO: 26) and F3NMBA (SEQ ID NO: 27) have an antisense DNA sequence. F3RFBS and F3RFBA have a sequence complementary to each other, and have XhoI and NcoI recognition sequences on both ends.

F3RFFS, F3RFBS, F3RFFA, F3RFBA, F3NMFS, F3NMBS, F3NMFA, and F3NMBA were synthesized by Pharmacia Biotech. F3RFFS and F3RFFA, and F3RFBS and F3RFBA were annealed, and were digested with BalI and XhoI, and NcoI and XhoI, respectively. They were introduced to the plasmid hATR5Hva/CVIDEC (BalI/NcoI) prepared by digesting with BalI and NcoI, and the nucleotide sequence was determined. The plasmid having the correct sequence was designated as hATR5Hvb/CVIDEC. The nucleotide sequence and the corresponding amino acid sequence of the humanized H chain version "b" contained in the plasmid hATR5Hvb/CVIDEC are shown in SEQ ID NO: 28. The amino

acid sequence of version "b" is also shown in SEQ ID NO: 29.

F3NMFS and F3NMFA, and F3NMBS and F3NMBA were annealed, and were digested with BalI and XhoI, and NcoI and XhoI, respectively. They were introduced to the plasmid hATR5Hva/CVIDEC (BalI/NcoI) prepared by digesting with BalI and NcoI, and the nucleotide sequence was determined. The plasmid having the correct sequence was designated as hATR5Hvc/CVIDEC. The nucleotide sequence and the corresponding amino acid sequence of the humanized H chain version "c" contained in the plasmid hATR5Hvc/CVIDEC are shown in SEQ ID NO: 30. The amino acid sequence of version "c" is also shown in SEQ ID NO: 31.

(iii) Construction of humanized H chain versions "d" and "e"

Versions "d" and "e" were generated by replacing the FR3 of version "a" with the FR3 derived from another human antibody using the FR-shuffling method. In order to replace the FR3 in version "d" with one derived from human antibody M62723 (DDBJ, Pascual V. et al., J. Clin. Invest., 86: 1320-1328, 1990), four DNA primers encoding the FR3 were generated. The FR-shuffling primer F3EPS (SEQ ID NO: 32) has a sense DNA sequence and F3EPA (SEQ ID NO: 33) has an antisense DNA sequence, and the 3'-end of the primers has a complementary sequence of 18 bp.

Exogenous primers F3PrS (SEQ ID NO: 34) and F3PrA (SEQ ID NO: 35) have a homology with the FR-shuffling primers F3EPS and F3EPA, and can also be used for other FR3's FR-shuffling. In order to replace the FR3 in version "e" with one derived from the human antibody Z80844 (DDBJ, Thomsett AR. et al., unpublished), two DNA primers encoding the FR3 were generated. The FR-shuffling primers F3VHS (SEQ ID NO: 36) has a sense DNA sequence and F3VHA (SEQ ID NO: 37) has an antisense DNA sequence, and the 3'-end of the primers has a complementary sequence of 18 bp. F3EPS, F3EPA, F3PrS,

F3PrA, F3VHS and F3VHA were synthesized by Pharmacia Biotech.

PCR was performed using the KOD DNA polymerase (Toyo Boseki) using the attached buffer under the condition of containing 5 μl each of 1 μM FR-shuffling primers F3EPS and F3EPA, or F3VHS and F3VHA, 0.2 mM dNTPs, 1.0 mM $MgCl_2$, and 2.5 units of KOD DNA polymerase in 100 μl of the reaction mixture, for 5 cycles at a temperature cycle of 94°C for 30 seconds, 50°C for 1 minute, and 74°C for 1 minute. After further addition of 100 pmole of exogenous primers F3PrS and F3PrA, PCR was performed for 25 cycles with the same temperature cycle.

DNA fragments amplified by the PCR method were separated by agarose gel electrophoresis using a 2% Nu Sieve GTG agarose (FMC BioProducts). The agarose strips containing about 424 bp long DNA fragments were excised, to which 3 volumes (ml/g) of TE was added, and then were extracted with phenol, phenol/chloroform, and chloroform to purify the DNA fragments. After precipitating the purified DNA with ethanol, one third the volume thereof was dissolved in 14 μl of water. The PCR reaction mixture obtained was digested with BalI and NcoI, and was introduced to the plasmid hATR5Hva/CVIDEC (BalI/NcoI) prepared by digesting with BalI and NcoI, and the nucleotide sequence was determined.

The plasmids having the correct sequence were designated as hATR5Hvd/CVIDEC and hATR5Hve/CVIDEC. The nucleotide sequence and the corresponding amino acid sequence of the humanized H chain version "d" contained in the plasmid hATR5Hvd/CVIDEC are shown in SEQ ID NO: 38, and the amino acid sequence of version "d" is also shown in SEQ ID NO: 39. The nucleotide sequence and the corresponding amino acid sequence of the humanized H chain version "e" contained in the plasmid hATR5Hve/CVIDEC are shown in SEQ ID NO: 40, and the amino acid sequence of version "e" is also shown in SEQ ID NO:

41.

(iv) Construction of humanized H chain versions "f" and "g"

Versions "f" and "g" were generated by replacing the FR3 of version "a" with the FR3 derived from another human antibody using the FR-shuffling method. In order to replace the FR3 in version "f" with one derived from human antibody L04345 (DDBJ, Hillson JL. et al., J. Exp. Med., 178: 331-336, 1993) and to replace the FR3 in version "g" with one derived from human antibody S78322 (DDBJ, Bejcek BE. et al., Cancer Res., 55: 2346-2351, 1995), two primers each encoding the FR3 were synthesized. The FR-shuffling primer F3SSS (SEQ ID NO: 42) of version "f" has a sense DNA sequence and F3SSA (SEQ ID NO: 43) has an antisense DNA sequence, and the 3'-end of the primers has a complementary sequence of 18 bp.

F3CDS (SEQ ID NO: 44) of version "g" has a sense DNA sequence and F3CDA (SEQ ID NO: 45) has an antisense DNA sequence, and the 3'-end of the primers has a complementary sequence of 18 bp. F3SSS, F3SSA, F3CDS, and F3CDA were synthesized and purified by Pharmacia Biotech. PCR was performed using the KOD DNA polymerase (Toyo Boseki) using the attached buffer under the condition of containing 5 µl each of 1 µM FR-shuffling primers F3SSS and F3SSA, or F3CDS and F3CDA, 0.2 mM dNTPs, 1.0 mM $MgCl_2$, and 2.5 units of KOD DNA polymerase in 100 µl of the reaction mixture, for 5 cycles at a temperature cycle of 94°C for 30 seconds, 50°C for 1 minute, and 74°C for 1 minute. After further addition of 100 pmole of exogenous primers F3PrS and F3PrA, PCR was performed for 25 cycles with the same temperature cycle.

DNA fragments amplified by the PCR method were separated by agarose gel electrophoresis using a 2% NuSieve GTG agarose (FMC BioProducts). The agarose strips containing about 424 bp long DNA fragments were

excised, to which 3 volumes (ml/g) of TE was added, and then were extracted with phenol, phenol/chloroform, and chloroform to purify the DNA fragments. After precipitating the purified DNA with ethanol, one third the volume thereof was dissolved in 14 μl of water. The PCR reaction mixture obtained was digested with BalI and NcoI, and was introduced to the plasmid hATR5Hva/CVIDEC (BalI/NcoI) prepared by digesting with BalI and NcoI, and the nucleotide sequence was determined.

The plasmids having the correct sequence were designated as hATR5Hvf/CVIDEC and hATR5Hvg/CVIDEC. The nucleotide sequence and the corresponding amino acid sequence of the humanized H chain version "f" contained in the plasmid hATR5Hvf/CVIDEC, and the amino acid sequence of version "f" are shown in SEQ ID NO: 46 and 47. The nucleotide sequence and the corresponding amino acid sequence of the humanized H chain version "g" contained in the plasmid hATR5Hvg/CVIDEC, and the amino acid sequence of version "g" are shown in SEQ ID NO: 48 and 49.

(v) Construction of the humanized H chain version "h"

Version "h" was generated by replacing the FR3 of version "a" with the FR3 derived from another human antibody using the FR-shuffling method. In order to replace the FR3 in version "h" with one derived from the human antibody Z26827 (DDBJ, van Der Stoep et al., J. Exp. Med., 177: 99-107, 1993), two primers each encoding the FR3 were synthesized. The FR-shuffling primer F3ADS (SEQ ID NO: 50) of version "h" has a sense DNA sequence and F3ADA (SEQ ID NO: 51) has an antisense DNA sequence, and the 3'-end of the primers has a complementary sequence of 18 bp.

F3ADS and F3ADA were synthesized and purified by Pharmacia Biotech. PCR was performed using the KOD DNA polymerase (Toyo Boseki) using the attached buffer under

the condition of containing 5 μl each of 1 μM FR-shuffling primers F3ADS and F3ADA, 0.2 mM dNTPs, 1.0 mM $MgCl_2$, and 2.5 units of KOD DNA polymerase in 100 μl of the reaction mixture, for 5 cycles at a temperature cycle of 94°C for 30 seconds, 50°C for 1 minute, and 74C for 1 minute. After further addition of 100 pmole of exogenous primers F3PrS and F3PrA, PCR was performed for 25 cycles with the same temperature cycle. DNA fragments amplified by the PCR method were separated by agarose gel electrophoresis using a 2% NuSieve GTG agarose (FMC BioProducts).

The agarose strips containing about 424 bp long DNA fragments were excised, to which 3 volumes (ml/g) of TE was added, and then were extracted with phenol, phenol/chloroform, and chloroform to purify the DNA fragments. After precipitating the purified DNA with ethanol, one third the volume thereof was dissolved in 14 μl of water. The PCR reaction mixture obtained was digested with BalI and NcoI, and was introduced to the plasmid hATR5Hva/CVIDEC (BalI/NcoI) prepared by digesting with BalI and NcoI, and the nucleotide sequence was determined. The plasmids having the correct sequence were designated as hATR5Hvh/CVIDEC. The nucleotide sequence and the corresponding amino acid sequence of the humanized H chain version "h" contained in the plasmid hATR5Hvh/CVIDEC, and the amino acid sequence of version "h" are shown in SEQ ID NO: 52. The amino acid sequence of version "h" is shown in SEQ ID NO: 53.

(vi) Construction of humanized H chain versions "i" and "j"

Versions "i" and "j" were generated by replacing the FR3 of version "a" with the FR3 derived from another human antibody using the FR-shuffling method. In order to replace the FR3 in version "i" with one derived from the human antibody U95239 (DDBJ, Manheimer-Lory AAJ., unpublished) and to replace the FR3 in version "j" with

one derived from the human antibody L03147 (DDBJ, Collect TA. et al., Proc. Natl. Acad. Sci. USA, 89: 10026-10030, 1992), two primers each encoding the FR3 were synthesized. The FR-shuffling primer F3MMS (SEQ ID NO: 54) of version "i" has a sense DNA sequence and F3MMA (SEQ ID NO: 55) has an antisense DNA sequence, and the 3'-end of the primers has a complementary sequence of 18 bp.

F3BMS (SEQ ID NO: 56) of version "j" has a sense DNA sequence and F3BMA (SEQ ID NO: 57) has an antisense DNA sequence, and the 3'-end of the primers has a complementary sequence of 18 bp. F3MMS, F3MMA, F3BMS, and F3BMA were synthesized and purified by Pharmacia Biotech. PCR was performed using the Ampli Taq Gold (Perkin-Elmer) using the attached buffer under the condition of containing 5 μl each of 1 μM FR-shuffling primers F3MMS and F3MMA, or F3BMS and F3BMA, 0.2 mM dNTPs, 1.0 mM $MgCl_2$, and 2.5 units of Ampli Taq Gold in 100 μl of the reaction mixture, for 5 cycles at a temperature cycle of 94°C for 30 seconds, 50°C for 1 minute, and 74°C for 1 minute. After further addition of 100 pmole of exogenous primers F3PrS and F3PrA, PCR was performed for 25 cycles with the same temperature cycle.

DNA fragments amplified by the PCR method were separated by agarose gel electrophoresis using a 2% Nu Sieve GTG agarose (FMC BioProducts). The agarose strips containing about 424 bp long DNA fragments were excised, to which 3 volumes (ml/g) of TE was added, and then were extracted with phenol, phenol/chloroform, and chloroform to purify the DNA fragments. After precipitating the purified DNA with ethanol, one third the volume thereof was dissolved in 14 μl of water. The PCR reaction mixture obtained was digested with BalI and NcoI, and was introduced to the plasmid hATR5Hva/CVIDEC (BalI/NcoI) prepared by digesting with BalI and NcoI, and the nucleotide sequence was determined.

The plasmids having the correct sequence were designated as hATR5Hvi/CVIDEC and hATR5Hvj/CVIDEC. The nucleotide sequence and the corresponding amino acid sequence of the humanized H chain version "i" contained in the plasmid hATR5Hvi/CVIDEC, and the amino acid sequence of version "i" are shown in SEQ ID NO: 58 and 59. The nucleotide sequence and the corresponding amino acid sequence of the humanized H chain version "j" contained in the plasmid hATR5Hvj/CVIDEC, and the amino acid sequence of version "j" are shown in SEQ ID NO: 60 and 61.

(vii) Construction of humanized H chain versions "b1" and "d1"

Versions "b1" and "d1" were generated by replacing the FR2 of versions "b" and "d" with the FR2 derived from another human antibody using the FR-shuffling method. In order to replace the FR2 with one derived from the human antibody P01742 (SWISS-PROT, Cunningham BA. et al., Biochemistry, 9: 3161-3170, 1970), two DNA primers encoding the FR2 were synthesized. The FR-shuffling vector F2MPS (SEQ ID NO: 62) has a sense DNA sequence and F2MPA (SEQ ID NO: 63) has an antisense DNA sequence. They also have a sequence complementary to each other, and have recognition sequences of EcoT221 and BalI on both ends thereof.

F2MPS and F2MPA were synthesized and purified by Pharmacia Biotech. F2MPS and F2MPA were annealed and were digested with EcoT22I and BalI. They were introduced to plasmids hATR5Hvb/CVIDEC (EcoT22I/BalI) and hATR5Hvd/CVIDEC (EcoT22I/BalI) prepared by digesting with EcoT22I and BalI, and the nucleotide sequence was determined. The plasmids having the correct sequence were designated as hATR5Hvb1/CVIDEC and hATR5Hvd1/CVIDEC. The nucleotide sequence and the corresponding amino acid sequence of the humanized H chain version "b1" contained in the plasmid hATR5Hvb1/CVIDEC, and the amino acid sequence of version "b1" are shown in SEQ ID NO: 64 and

65. The nucleotide sequence and the corresponding amino acid sequence of the humanized H chain version "d1" contained in the plasmid hATR5Hvd1/CVIDEC, and the amino acid sequence of version "d1" are shown in SEQ ID NO: 66 and 67.

(viii) Construction of humanized H chain versions "b3" and "d3"

Versions "b3" and "d3" were generated by replacing the FR2 of versions "b" and "d" with the FR2 derived from another human antibody using the FR-shuffling method. In order to replace the FR2 with one derived from the human antibody Z80844 (DDDJ, Thomsett AR. et al., unpublished), two DNA primers encoding the FR2 were synthesized. The FR-shuffling vector F2VHS (SEQ ID NO: 68) has a sense DNA sequence and F2VHA (SEQ ID NO: 69) has an antisense DNA sequence. They also have a sequence complementary to each other, and have recognition sequences of EcoT221 and BalI on both ends thereof. The synthesis and purification of F2VHS and F2VHA was referred to Pharmacia Biotech.

F2VHS and F2VHA were annealed and were digested with EcoT22I and BalI. They were introduced to plasmids hATR5Hvb/CVIDEC (EcoT22I/BalI) and hATR5Hvd/CVIDEC (EcoT22I/BalI) prepared by digesting with EcoT22I and BalI, and the nucleotide sequence was determined. The plasmids having the correct sequence were designated as hATR5Hvb3/CVIDEC and hATR5Hvd3/CVIDEC. The nucleotide sequence and the corresponding amino acid sequence of the humanized H chain version "b3" contained in the plasmid hATR5Hvb3/CVIDEC, and the amino acid sequence of version "b3" are shown in SEQ ID NO: 70 and 71. The nucleotide sequence and the corresponding amino acid sequence of the humanized H chain version "d3" contained in the plasmid hATR5Hvd3/CVIDEC, and the amino acid sequence of version "d3" are shown in SEQ ID NO: 72 and 73.

(2) Construction of a humanized antibody L chain V region

(i) version "a"

The humanized ATR-5 antibody L chain V region was generated by the CDR-grafting using the PCR method. For the generation of a humanized antibody L chain (version "a") having framework regions derived from human antibody Z37332 (DDBJ, Welschof M. et al., J. Immunol. Methods, 179: 203-214, 1995), seven PCR primers were used.

CDR-grafting primers h5Lv1S (SEQ ID NO: 74) and h5Lv4S (SEQ ID NO: 75) have a sense DNA sequence, CDR-grafting primers h5Lv2A (SEQ ID NO: 76), h5Lv3A (SEQ ID NO: 77), and h5Lv5A (SEQ ID NO: 78) have an antisense DNA sequence, and each primer has 20 bp complementary sequences on both ends thereof. Exogenous primers h5LvS (SEQ ID NO: 79) and h5LvA (SEQ ID NO: 80) have a homology with CDR-grafting primers h5Lv1S and h5Lv5A. The synthesis and purification of CDR-grafting primers h5Lv1S, h5Lv4S, h5Lv2A, h5Lv3A, h5Lv5A, h5LvS, and h5LvA were referred to Pharmacia Biotech.

The PCR solutions contain, in 100 µl, 120 mM Tris-HCl (pH 8.0), 10 mM KCl, 6 mM $(NH_4)_2SO_4$, 0.1% Triton X-100, 0.001% BSA, 0.2 mM dNTPs (dATP, dGTP, dCTP, dTTP), 1 mM $MgCl_2$, 2.5 units of KOD DNA polymerase (Toyo Boseki), 50 pmole of the CDR-grafting primers h5Lv1S, h5Lv2A, h5Lv3A, h5Lv4S, and h5Lv5A.

PCR was performed using the DNA Thermal Cycler 480 (Perkin-Elmer) for 5 cycles with the temperature cycle of 94°C for 30 seconds, 50°C for 1 minute, and 72°C for 1 minute to assemble 5 CDR-grafting primers. After further addition of 100 pmole of exogenous primers h5LvS and h5LvA to the reaction mixture, PCR was performed for 30 cycles with the temperature cycle of 94°C for 30 seconds, 52°C for 1 minute, and 72°C for 1 minute to amplify the assembled DNA fragments.

The PCR reaction mixture was separated by agarose gel electrophoresis using a 3% NuSieve GTG agarose (FMC BioProducts), and the agarose strips containing about 400 bp long DNA fragments were excised. The agarose strips

were extracted with phenol and chloroform, DNA fragments were recovered by ethanol precipitation. The recovered DNA fragments were digested with the restriction enzymes SplI (Takara Shuzo) and BglII (Takara Shuzo) at 37°C for 4 hours. The digestion mixture was extracted with phenol and chloroform, and after the ethanol precipitation of the DNA fragments, they were dissolved in 10 μl of TE. The SplI-BglII DNA fragment prepared as above encoding the humanized L chain V region and the CVIDEC vector prepared by digesting with SplI and BglII were ligated using the DNA ligation kit ver.2 (Takara Shuzo) by reacting at 16°C for 1 hour according to the instructions attached to the kit.

The ligation mixture was added to 100 μl of E. coli JM109 competent cells (Nippongene) and was incubated for 30 minutes on ice and for 1 minute at 42°C. Then, 300 μl of the Hi-Competence Broth (Nippongene) was added thereto, incubated at 37°C for 1 hour, and then the E. coli was plated on the LBA agar medium and incubated overnight at 37°C to obtain an E. coli transformant. The transformant was cultured overnight in 3 ml of the LBA medium, and from the cell fractions, plasmid DNA was prepared using the QIAprep Spin Plasmid Kit (QIAGEN).

The nucleotide sequence of the cDNA coding region in the plasmid was determined using the Dye Terminator Cycle Sequencing FS Ready Reaction Kit (Perkin-Elmer) by the DNA Sequencer 373A (Perkin-Elmer). As the sequencing primer, M13 Primer M4 (Takara Shuzo) and M13 Primer RV (Takara Shuzo) were used, and the sequence was determined by confirming the nucleotide sequence in both directions. The plasmid that contains the gene encoding the humanized antibody L chain V region and that has a BglII recognition sequence and the Kozak sequence at the 5'-end, and a SplI recognition sequence at the 3'-end was designated as hATR5Lva/CVIDEC. The nucleotide sequence (including the corresponding amino acid sequence) of the

humanized L chain version "a" is shown in SEQ ID NO: 81. The amino acid sequence of version "a" is also shown in SEQ ID NO: 82.

(ii) Versions "b" and "c"

Versions "b" and "c" were generated by replacing (FR-shuffling) the FR3 of version "a". For version "b" the FR3 derived from human antibody S68699 (DDBJ, Hougs L. et al., Exp. Clin. Immunogen et., 10: 141-151, 1993) was used, and for version "c" the FR3 derived from human antibody P01607 (SWISS-PROT, Epp O et al., Biochemistry, 14: 4943-4952, 1975) was used, respectively.

Primers F3SS (SEQ ID NO: 83) and F3SA (SEQ ID NO: 84) encoding the FR3 of version "b", or primers F3RS (SEQ ID NO: 85) and F3RA (SEQ ID NO: 86) encoding the FR3 of version "c" have a sequence complementary to each other, and have the recognition sequences of the restriction enzymes KpnI and PstI on both ends thereof. The synthesis and purification of F3SS, F3SA, F3RS, and F3RA were referred to Pharmacia Biotech. 100 pmole each of F3SS and F3SA, or F3RS and F3RA were annealed by treating at 96°C for 2 minutes and at 50°C for 2 minutes and the double stranded DNA fragments were generated.

These double stranded DNA fragments were digested with the restriction enzyme KpnI (Takara Shuzo) at 37°C for 1 hour, and then with the restriction enzyme PstI (Takara Shuzo) at 37°C for 1 hour. The digestion mixture was extracted with phenol and chloroform, and after it was precipitated with ethanol, it was dissolved in TE.

The plasmid hATR5Lva/CVIDEC was digested with the restriction enzyme KpnI (Takara Shuzo) at 37°C for 1 hour, and then with the restriction enzyme PstI (Takara Shuzo) at 37°C for 1 hour. The digestion mixture was separated by agarose gel electrophoresis using a 1.5% NuSieve GTG agarose (FMC BioProducts), and the agarose strips having about 3000 bp long DNA fragments were excised. The agarose strip was extracted with phenol and chloroform, and after the DNA fragments were precipitated

with ethanol, they were dissolved in TE.

The KpnI-PstI DNA fragment prepared as above encoding the FR3 of versions "b" or "c" and the hATR5Lva/CVIDEC vector in which the FR3 was removed by digesting with KpnI and PstI were ligated using the DNA ligation kit ver.2 (Takara Shuzo) by reacting at 16°C for 1 hour according to the instructions attached to the kit.

The ligation mixture was added to 100 µl of E. coli JM109 competent cells (Nippongene) and was incubated for 30 minutes on ice and for 1 minute at 42°C. Then, 300 µl of the Hi-Competence Broth (Nippongene) was added thereto, incubated at 37°C for 1 hour, and then the E. coli was plated on the LBA agar medium and incubated overnight at 37°C to obtain an E. coli transformant. The transformant was cultured overnight in 3 ml of the LBA medium, and from the cell fractions, plasmid DNA was prepared using the QIAprep Spin Plasmid Kit (QIAGEN).

The nucleotide sequence of the cDNA coding region in the plasmid was determined using the Dye Terminator Cycle Sequencing FS Ready Reaction Kit (Perkin-Elmer) by the DNA Sequencer 373A (Perkin-Elmer). As the sequencing primer, M13 Primer M4 (Takara Shuzo) and M13 Primer RV (Takara Shuzo) were used, and the sequence was determined by confirming the nucleotide sequence in both directions.

The plasmids that contain the gene encoding version "b" or version "c" in which the FR3 of humanized antibody L chain version "a" was replaced was designated as hATR5Lvb/CVIDEC or hATR5Lvc/CVIDEC, respectively. The nucleotide sequence and the corresponding amino acid sequence of the humanized L chain version "b" contained in plasmid hATR5Lvb/CVIDEC and the amino acid sequence of version "b" are shown in SEQ ID NO: 87 and 88. The nucleotide sequence and the corresponding amino acid sequence of the humanized L chain version "c" contained in plasmid hATR5Lvc/CVIDEC and the amino acid sequence of version "c" are shown in SEQ ID NO: 89 and 90.

(iii) Versions "b1" and "b2"

Versions "b1" and "b2" were generated by replacing the FR2 of version "b". For version "b1" the FR2 derived from human antibody S65921 (DDBJ, Tonge DW et al., Year Immunol., 7: 56-62, 1993) was used, and for version "b2" the FR2 derived from human antibody X93625 (DDBJ, Cox JP et al., Eur. J. Immunol., 24: 827-836, 1994) was used, respectively.

Primers F2SS (SEQ ID NO: 91) and F2SA (SEQ ID NO: 92) encoding the FR2 of version "b1", or primers F2XS (SEQ ID NO: 93) and F2XA (SEQ ID NO: 94) encoding the FR2 of version "b2" have a sequence complementary to each other, and have the recognition sequences of the restriction enzymes AflII and SpeI on both ends thereof. F2SS, F2SA, F2XS, and F2XA were synthesized by Pharmacia Biotech. 100 pmole each of F2SS and F2SA, or F2XS and F2XA were annealed by treating at 96°C for 2 minutes and at 50°C for 2 minutes, and the double stranded DNA fragments were generated.

These double stranded DNA fragments were digested with the restriction enzymes AflII (Takara Shuzo) and SpeI (Takara Shuzo) at 37°C for 1 hour. The digestion mixture was extracted with phenol and chloroform, and after the DNA fragments were precipitated with ethanol, they were dissolved in TE.

The plasmid hATR5Lvb/CVIDEC was digested with the restriction enzymes AflII (Takara Shuzo) and SpeI (Takara Shuzo) at 37°C for 1 hour. The digestion mixture was separated by agarose gel electrophoresis using a 1.5% NuSieve GTG agarose (FMC BioProducts), and the agarose strips having about 3000 bp long DNA fragments were excised. The agarose strip was extracted with phenol and chloroform, and after the DNA fragments were precipitated with ethanol, they were dissolved in TE.

The AflII-SpeI DNA fragment prepared as above encoding the FR2 of version "b1" or "b2" and the hATR5Lvb/CVIDEC vector in which the FR2 was removed by

digesting with AflII and SpeI were ligated using the DNA ligation kit ver.2 (Takara Shuzo) by reacting at 16°C for 1 hour according to the instructions attached to the kit.

The ligation mixture was added to 100 µl of E. coli JM109 competent cells (Nippongene) and was incubated for 30 minutes on ice and for 1 minute at 42°C. Then, 300 µl of the Hi-Competence Broth (Nippongene) was added thereto, incubated at 37°C for 1 hour, and then the E. coli was plated on the LBA agar medium and incubated overnight at 37°C to obtain an E. coli transformant. The transformant was cultured overnight at 37°C in 4 ml of the LBA medium, and from the cell fractions, plasmid DNA was prepared using the QIAprep Spin Plasmid Kit (QIAGEN).

The nucleotide sequence of the cDNA coding region in the plasmid was determined using the Dye Terminator Cycle Sequencing FS Ready Reaction Kit (Perkin-Elmer) by the DNA Sequencer 373A (Perkin-Elmer). As the sequencing primer, M13 Primer M4 (Takara Shuzo) and M13 Primer RV (Takara Shuzo) were used, and the sequence was determined by confirming the nucleotide sequence in both directions.

The plasmids that contain the gene encoding version "b1" or "b2" in which the FR2 of humanized antibody L chain version "b" was replaced was designated as hATR5Lvb1/CVIDEC and hATR5Lv2/CVIDEC, respectively. The nucleotide sequence and the corresponding amino acid sequence of the humanized L chain version "b1" contained in plasmid hATR5Lvb1/CVIDEC and the amino acid sequence of version "b1" are shown in SEQ ID NO: 95 and 96. The nucleotide sequence and the corresponding amino acid sequence of the humanized L chain version "b2" contained in plasmid hATR5Lvb2/CVIDEC and the amino acid sequence of version "b2" are shown in SEQ ID NO: 97 and 98.

(3) Construction of the expression vector of humanized antibody

(i) Combination of humanized H chain ad chimeric L chain

The plasmid hATR5Hva/CVIDEC containing a H chain V region was digested with NheI and SalI, and a cDNA fragment of the humanized H chain V region was recovered and introduced to chATR5/N5KG4P (SalI/NheI) prepared by digesting chATR5/N5KG4P, a chATR-5 antibody expression plasmid vector, with NheI and SalI. The plasmid thus generated was designated as hHva-chLv/N5KG4P.

The plasmid hATR5Hvb/CVIDEC containing a H chain V region was digested with NheI and SalI, and a cDNA fragment of the humanized H chain V region was recovered and introduced to chATR5/N5KG4P (SalI/NheI) prepared by digesting chATR5/N5KG4P, a chATR-5 antibody expression plasmid vector, with NheI and SalI. The plasmid thus generated was designated as hHvb-chLv/N5KG4P.

The plasmids hATR5Hvc/CVIDEC, hATR5Hvd/CVIDEC, and hATR5Hve/CVIDEC containing a H chain V region were digested with NheI and SalI, and cDNA fragments of the humanized H chain V region were recovered and introduced to chATR5/N5KG4P (SalI/NheI) prepared by digesting chATR5/N5KG4P, a chATR-5 antibody expression plasmid vector, with NheI and SalI. The plasmids thus generated were designated as hHvc-chLv/N5KG4P, hHvd-chLv/N5KG4P, and hHve-chLv/N5KG4P.

The plasmids hATR5Hvf/CVIDEC and hATR5Hvh/CVIDEC containing a H chain V region were digested with NheI and SalI, and cDNA fragments of the humanized H chain V region were recovered and introduced to chATR5/N5KG4P (SalI/NheI) prepared by digesting chATR5/N5KG4P, a chATR-5 antibody expression plasmid vector, with NheI and SalI. The plasmids thus generated were designated as hHvf-chLv/N5KG4P and hHvh-chLv/N5KG4P.

The plasmids hATR5Hvi/CVIDEC and hATR5Hvj/CVIDEC containing a H chain V region were digested with NheI and SalI, and cDNA fragments of the humanized H chain V region were recovered and introduced to chATR5/N5KG4P (SalI/NheI) prepared by digesting chATR5/N5KG4P, a chATR-5 antibody expression plasmid vector, with NheI and SalI.

The plasmids thus generated were designated as hHvi-chLv/N5KG4P and hHvj-chLv/N5KG4P.

The plasmids hATR5Hb1/CVIDEC and hATR5Hvd1/CVIDEC containing a H chain V region were digested with NheI and SalI, and cDNA fragments of the humanized H chain V region were recovered and introduced to chATR5/N5KG4P (SalI/NheI) prepared by digesting chATR5/N5KG4P, a chATR-5 antibody expression plasmid vector, with NheI and SalI. The plasmids thus generated were designated as hHvb1-chLv/N5KG4P and hHvd1-chLv/N5KG4P.

(ii) Combination of humanized L chain ad chimeric H chain

Using an antibody expression vector N5KG4P, it was combined with a chimeric H chain and was expressed, and the humanized L chain was evaluated.

The plasmids hATR5Lva/CVIDEC, hATR5Lvb/CVIDEC, hATR5Lvc/CVIDEC, hATR5Lvb1/CVIDEC, and hATR5Lvb2/CVIDEC were digested with the restriction enzymes BglII (Takara Shuzo) and SplI (Takara Shuzo) at 37°C for 2-3 hours. The digestion mixture was separated by agarose gel electrophoresis using a 1.5% or 2% NuSieve GTG agarose (FMC BioProducts), and the agarose strips having about 400 bp long DNA fragments were excised. The agarose strips were extracted with phenol and chloroform, and after the DNA fragments were precipitated with ethanol, they were dissolved in TE.

The SplI-BglII DNA fragment containing the gene encoding the a humanized L chain V region of each of these versions and the hATR5Hv/N5KG4P digested with SplI and BglII were ligated using the DNA ligation kit ver.2 (Takara Shuzo) by reacting at 16°C for 1 hour according to the instructions attached to the kit.

The ligation mixture was added to 100 µl of E. coli JM109 competent cells (Nippongene) and was incubated for 30 minutes on ice and for 1 minute at 42°C. Then, 300 µl of the Hi-Competence Broth (Nippongene) was added

thereto, incubated at 37°C for 1 hour, and then the E. coli was plated on the LBA agar medium and incubated overnight at 37°C to obtain an E. coli transformant.

The transformant was cultured overnight at 37°C in 250 ml or 500 ml of the LBA medium, and from the cell fractions, plasmid DNA was prepared using the Plasmid Maxi Kit (QIAGEN). The plasmids in which a gene encoding the chimeric H chain and humanized L chain was introduced were designated as chHv-hLva/N5KG4P, chHv-hLvb/N5KG4P, chHv-hLvc/N5KG4P, chHv-hLvb1/N5KG4P, and chHv-hLvb2/N5KG4P.

(iii) Combination of humanized H chain and humanized L chain

The plasmid hATR5Hva/CVIDEC containing a H chain V region was digested with NheI and SalI, and a cDNA fragment of the humanized H chain V region was recovered and introduced to hLva/N5KG4P (SalI/NheI) prepared by digesting plasmid chHv-hLva/N5KG4P containing the cDNA sequence of humanized ATR-5 antibody L chain version "a" with NheI and SalI. The plasmid thus generated was designated as hHva-hLva/N5KG4P.

The plasmids hATR5Hvb/CVIDEC and hATR5Hvc/CVIDEC containing a H chain V region were digested with NheI and SalI, and cDNA fragments of the humanized H chain V region were recovered and introduced to hLva/N5KG4P (SalI/NheI) prepared by digesting plasmid chHv-hLva/N5KG4P containing the cDNA sequence of humanized ATR-5 antibody L chain version "a" with NheI and SalI. The plasmids thus generated were designated as hHvb-hLva/N5KG4P and hHvc-hLva/N5KG4P.

The plasmids hATR5Hvb/CVIDEC, hATR5Hvd/CVIDEC, and hATR5Hve/CVIDEC containing a H chain V region were digested with NheI and SalI, and cDNA fragments of the humanized H chain V region were recovered and introduced to hLvb/N5KG4P (SalI/NheI) prepared by digesting plasmid chHv-hLvb/N5KG4P containing the cDNA sequence of humanized ATR-5 antibody L chain version "b" with NheI

and SalI. The plasmids thus generated were designated as hHvb-hLvb/N5KG4P, hHvd-hLvb/N5KG4P, and hHve-hLvb/N5KG4P.

The plasmids hATR5Hvf/CVIDEC, hATR5Hvg/CVIDEC, and hATR5Hvh/CVIDEC containing a H chain V region were digested with NheI and SalI, and cDNA fragments of the humanized H chain V region were recovered and introduced to hLvb/N5KG4P (SalI/NheI) prepared by digesting plasmid chHv-hLvb/N5KG4P containing the cDNA sequence of humanized ATR-5 antibody L chain version "b" with NheI and SalI. The plasmids thus generated were designated as hHvf-hLvb/N5KG4P, hHvg-hLvb/N5KG4P, and hHvh-hLvb/N5KG4P.

The plasmids hATR5Hvi/CVIDEC and hATR5Hvj/CVIDEC containing a H chain V region were digested with NheI and SalI, and cDNA fragments of the humanized H chain V region were recovered and introduced to hLvb/N5KG4P (SalI/NheI) prepared by digesting plasmid chHv-hLvb/N5KG4P containing the cDNA sequence of humanized ATR-5 antibody L chain version "b" with NheI and SalI. The plasmids thus generated were designated as hHvi-hLvb/N5KG4P and hHvj-hLvb/N5KG4P.

The plasmids hATR5Hvb1/CVIDEC and hATR5Hvd1/CVIDEC containing a H chain V region were digested with NheI and SalI, and cDNA fragments of the humanized H chain V region were recovered and introduced to hLvb/N5KG4P (SalI/NheI) prepared by digesting plasmid chHv-hLvb/N5KG4P containing the cDNA sequence of humanized ATR-5 antibody L chain version "b" with NheI and SalI. The plasmids thus generated were designated as hHvb1-hLvb/N5KG4P and hHvd1-hLvb/N5KG4P.

The plasmids hATR5Hvb3/CVIDEC and hATR5Hvd3/CVIDEC containing a H chain V region were digested with NheI and SalI, and cDNA fragments of the humanized H chain V region were recovered and introduced to hLvb/N5KG4P (SalI/NheI) prepared by digesting plasmid chHv-hLvb/N5KG4P containing the cDNA sequence of humanized ATR-5 antibody L chain version "b" with NheI and SalI. The plasmids thus generated were designated as hHvb3-

hLvb/N5KG4P and hHvd3-hLvb/N5KG4P.

The plasmid hATR5Hvb/CVIDEC containing a H chain V region was digested with NheI and SalI, and a cDNA fragment of the humanized H chain V region was recovered and introduced to hLvb1/N5KG4P (SalI/NheI) and hLvb2/N5KG4P (SalI/NheI) prepared by digesting plasmids chHv-hLvb1/N5KG4P and chHv-hLvb2/N5KG4P containing the cDNA sequence of humanized ATR-5 antibody L chain versions "b1" and "b2" with NheI and SalI. The plasmids thus generated were designated as hHvb-hLvb1/N5KG4P and hHvb-hLvb2/N5KG4P.

The plasmid hATR5Hvi/CVIDEC containing a H chain V region was digested with NheI and SalI, and a cDNA fragment of the humanized H chain V region was recovered and introduced to hLvb1/N5KG4P (SalI/NheI) and hLvb2/N5KG4P (SalI/NheI) prepared by digesting plasmids chHv-hLvb1/N5KG4P and chHv-hLvb2/N5KG4P containing the cDNA sequence of humanized ATR-5 antibody L chain versions "b1" and "b2" with NheI and SalI. The plasmids thus generated were designated as hHvi-hLvb1/N5KG4P and hHvi-hLvb2/N5KG4P.

(4) Transfection into COS-7 cells

In order to evaluate the activity of binding to the antigen and neutralizing activity of humanized antibody, the above antibody was transiently expressed in COS-7 cells.

The constructed expression plasmid vector was transduced into COS-7 cells by electroporation using the Gene Pulser instrument (Bio Rad). Fifty μg or 20 μg of the plasmid was added to 0.78 ml of COS-7 cells suspended in PBS at a cell concentration of 1×10^7 cells/ml, which was subjected to pulses of 1,500 V and 25 μF capacity.

After 10 minutes of the recovery period at room temperature, the electroporated cells were suspended in a DMEM medium (GIBCO) containing 5% Ultra low IgG fetal bovine serum (GIBCO), and cultured using a 10 cm culture

dish or 15 cm culture dish in a 5% CO_2 incubator. After culturing for 24 hours, the culture supernatant was aspirated off, and then a serum-free medium HBCHO (Irvine Scientific) was added. After further culturing for 72 hours or 96 hours, the culture supernatant was collected and centrifuged to remove cell debris.

(5) Purification of antibody

From the culture supernatant of the COS-7 cells, the antibody was purified using the AffiGel Protein A MAPSII kit (Bio Rad) or the rProtein A Sepharose Fast Flow (Pharmacia Biotech). Purification using the AffiGel Protein A MAPSII kit was carried out according to the instructions attached to the kit. Purification using the rProtein A Sepharose Fast Flow was carried out as follows:

One ml of rProtein A Sepharose Fast Flow was filled into a column and the column was equilibrated by 10 volumes of TBS. The culture supernatant of COS-7 cells was applied to the equilibrated column, which was then washed with 10 volumes of TBS. The adsorbed antibody fraction was eluted by 13.5 ml of 2.5 mM HCl (pH 3.0). The eluate was neutralized by adding 1.5 ml of 1 M Tris-HCl (pH 8.0).

By performing ultrafiltration two or three times for the purified antibody fraction using the Centriprep 30 or 100 (amicon), the solvent was replaced to TBS, and was finally concentrated to about 1.5 ml.

Reference Example 6. Antibody quantitation and activity evaluation

(1) Measurement of antibody concentration by ELISA

ELISA plates for measurement of antibody concentration were prepared as follows: Each well of a 96-well ELISA plate (Maxisorp, NUNC) was immobilized by 100 μl of goat anti-human IgGγ antibody (BIO SOURCE) prepared to a concentration of 1 μg/ml in the immobilization buffer (0.1 M $NaHCO_3$, 0.02% NaN_3, pH 9.6)

(hereinafter referred to as CB). After blocking with 200 µl of the dilution buffer (50 mM Tris-HCl, 1 mM $MgCl_2$, 0.1 M NaCl, 0.05% Tween 20, 0.02% NaN_3, 1% bovine serum albumin (BSA), pH 8.1) (hereinafter referred to as DB), the culture supernatant of the COS-7 cells in which antibody was expressed or purified antibody were serially diluted with DB, and then added to each well.

After incubating at room temperature for 1 hour followed by washing with the Dulbecco PBS containing 0.05% Tween 20 (hereinafter referred to as RB), 100 µl of alkaline phosphatase-conjugated goat anti-human IgGγ antibody (Biosource) which was diluted 1000-fold with DB was added. After incubating at room temperature for 1 hour followed by washing with the RB, Sigma104 (p-nitrophenyl phosphate, SIGMA) dissolved in the substrate buffer (50 mM $NaHCO_3$, 10 mM $MgCl_2$, pH 9.8) to 1 mg/ml was added, and then the absorbance at 405/655 nm was measured using the Microplate Reader (Bio Rad). As the standard for the measurement of concentration, IgG4κ (Binding Site) was used.

(2) Measurement of the activity of binding to the antigen

Cell ELISA plates for measurement of antigen binding were prepared as follows. Cells used were human bladder carcinoma cells J82 (ATCC HTB-1). To 60 wells of a 96-well cell culture plate, 1×10^5 J82 cells were inoculated. This was cultured (RPMI1640 medium containing 10% fetal bovine serum (GIBCO)) for one day in a CO_2 incubator to allow the cells to be attached thereto. After discarding the culture liquid, each well was washed twice with 300 µl PBS. 100 µl of PBS containing 4% paraformaldehyde (hereinafter referred to as PFA/PBS) was added to each well, and placed on ice for 10 minutes to immobilize the cells.

PFA/PBS was discarded, and each well was washed

twice with 300 μl of PBS, and then blocked with 250 μl of DB. The culture supernatant or purified antibody was serially diluted with DB, 100 μl of which was added to each well. After incubating at room temperature for 2 hours followed by washing with RB, 100 μl of alkaline phosphatase-conjugated goat anti-human IgGγ antibody (BioSource) diluted 1000-fold with DB was added. After incubating for 1 hour followed by washing with RB, the substrate solution was added, and then absorbance at 405/655 nm was measured using the Microplate Reader (Bio-Rad).

(3) Measurement of neutralizing activity

The neutralizing activity of mouse antibody, chimeric antibody, and humanized antibody was measured with the inhibiting activity against the Factor Xa-production activity by human placenta-derived thromboplastin, Thromborel S (Boehringer AG), as an index. Thus, 60 μl of the buffer (TBS containing 5 mM $CaCl_2$ and 0.1% BSA) was added to 10 μl of 1.25 mg/ml Thromborel S and 10 μl of appropriately diluted antibody, which was then incubated in a 96-well plate at room temperature for 1 hour. Ten μl each of 3.245 μg/ml human Factor X (Celsus Laboratories) and 82.5 ng/ml human Factor VIIa (Enzyme Research) were added thereto, and then were incubated at room temperature for 1 hour.

Ten μl of 0.5 M EDTA was added to stop the reaction, to which 50 μl of the chromogenic substrate solution was added and the absorbance at 405/655 nm was determined using the Microplate Reader (Bio Rad). After reacting at room temperature for 1 hour, the absorbance at 405/655 nm was determined again. The neutralizing activity may be determined by calculating the residual activity (%) from each change in absorbance with the hourly absorbance change at no antibody addition as a 100% activity.

The chromogenic substrate solution was prepared by dissolving the Testzyme chromogenic substrate S-2222 (Chromogenix) according to the attached instructions, diluting 2-fold with purified water and mixing with a polybrene solution (0.6 mg/ml hexadimethylene bromide, SIGMA) at 1:1.

(4) Evaluation of activity

(i) Combination of the humanized H chain version "a" and a chimeric L chain

An antibody (a-ch) which is the humanized H chain version "a" combined with a chimeric L chain was generated, and was tested for the binding activity to the antigen by the cell ELISA. The amount bound to the antigen was found to be decreased at the high concentration. The neutralizing activity against the antigen by FXa production-inhibition was weak as compared that of to the positive control chimeric antibody (ch-ch). Therefore, it was decided to perform the version-up of the humanized H chain by FR-shuffling. The chimeric antibody used herein was the one that was expressed in COS-7 cells, purified, and evaluated.

(ii) Combination of the humanized L chain version "a" and a chimeric H chain

An antibody (ch-a) which is the humanized L chain version "a" combined with a chimeric H chain was generated, and was tested for the binding activity to the antigen by the cell ELISA. It was found to have the binding activity equal to or higher than that of the chimeric antibody. On the other hand, the neutralizing activity against the antigen was weak as compared to that of the positive control chimeric antibody. Therefore, it was decided to perform the version-up of the humanized L chain by FR-shuffling. The chimeric antibody used herein was the one that was expressed in COS-7 cells, purified, and evaluated.

(iii) Combination of the humanized H chain version "a" and the humanized L chain version "a"

An antibody (a-a) which is the humanized H chain version "a" combined with the humanized L chain version "a" was generated, and was tested for the binding activity to the antigen by the cell ELISA. The amount bound to the antigen was found to be decreased in the high concentration side. The neutralizing activity against the antigen by FXa production-inhibition was weak as compared to that of the positive control chimeric antibody. Therefore, it was decided to perform the version-up of the humanized H chain and L chain by FR-shuffling. The chimeric antibody used herein was the one that was expressed in COS-7 cells, purified, and evaluated.

(iv) Combination of the humanized H chain versions "b", "c", and "d", and a chimeric L chain

Antibodies ("b-ch", "c-ch", and "d-ch", respectively) which are the humanized H chain subjected to version-up by FR-shuffling combined with a chimeric L chain were generated, and were tested for the binding activity to the antigen by the cell ELISA. "d-ch" exhibited a binding activity equal to that of the chimeric antibody, and "b-ch" and "c-ch" exhibited a slightly lower binding activity. On the other hand, the neutralizing activity against the antigen as compared to the that of positive control chimeric antibody was almost equal in "b-ch", and slightly weak in "d-ch". In version "c-ch", it was significantly weaker than that of the chimeric antibody. Therefore, the humanized H chain versions "b" and "d" were considered the ones of the humanized H chain to exhibit a high activity.

(v) Combination of the humanized H chain version "b" and the humanized L chain version "a"

An antibody (b-a) which is the humanized H chain version "b" subjected to version-up by FR-shuffling combined with the humanized L chain version "a" was generated, and was tested for the binding activity to the antigen by the cell ELISA. The amount bound to the

antigen was found to be decreased at the high concentration. On the other hand, the neutralizing activity against the antigen was significantly weak as compared to that of the positive control chimeric antibody. Therefore, "b-a" and "a-a" were the ones that exhibit a high activity. The chimeric antibody used herein was the one that was expressed in COS-7 cells, purified, and evaluated.

(vi) Combination of the humanized L chain versions "b" and "c", and a chimeric H chain

Antibodies ("ch-b" and "ch-c", respectively) which are the humanized L chain versions "b" and "c" combined with a chimeric H chain were generated, and both of them were found to have the binding activity to the antigen and the neutralizing activity against the antigen equal to the chimeric antibody. Therefore, versions "b" and "c" were chosen as a candidate for a humanized antibody L chain. Mouse antibody-derived version "b" which is one amino acid fewer in the amino acid residue number is considered to be superior to version "c" in terms of antigenicity. The chimeric antibody used herein was the one that was expressed in CHO cells DG44, purified, and evaluated. In the evaluation hereinafter the antibody was used as the positive control.

(vii) Combination of the humanized H chain version "b" and the humanized L chain versions "b" and "c"

Antibodies ("b-b" and "b-c", respectively) which are the humanized H chain version "b" combined with the humanized L chain versions "b" and "c" were generated, and tested for the binding activity to the antigen and the neutralizing activity against the antigen. Both of them had a slightly lower activity than that of the chimeric antibody in both the binding activity and the neutralizing activity.

(viii) Combination of the humanized H chain versions "b" and "d", and the humanized L chain

version "b"

Antibodies ("b-b" and "d-b", respectively) which are the humanized H chain subjected to version-up by FR-shuffling combined with the humanized L chain version "b" were generated, and were tested for the binding activity to the antigen by the cell ELISA. "d-b" exhibited a binding activity equal to that of the chimeric antibody, and "b-b" exhibited a slightly lower binding activity at the high concentration. On the other hand, the neutralizing activity against the antigen as compared to that of the positive control chimeric antibody was slightly low in "b-b", and significantly weak in "d-b". Therefore, it was shown that "b-b" is a high neutralizing activity version, whereas "d-b" is a high binding activity version.

(ix) Combination of the humanized H chain version "e", and a chimeric L chain and the humanized L chain version "b"

Antibodies ("e-ch" and "e-b", respectively) which are the humanized L chain version "e" combined with a chimeric L chain and the humanized version "b" were generated. "e-ch" exhibited a binding activity to the antigen equal to that of the chimeric antibody, but in "e-b" the amount of antibody expressed was very little and most of the binding activity was lost. The neutralizing activity against the antigen of "e-ch" was significantly low as compared to that of the chimeric antibody. Therefore, it was concluded that the H chain version "e" combined with L chain version "b" did not work well.

(x) Combination of the humanized H chain versions "f", "g", and "h", and the humanized L chain version "b"

Antibodies ("f-b", "g-b", and "h-b", respectively) which are the humanized H chain versions "f", "g", and "h" combined with the humanized L chain version "b" were generated. In "f-b" and "h-b" antibody, the amount of

antibody expressed was very little. For versions "f" and "h", antibodies combined with the chimeric L chain were generated, but were not expressed. "g-b" reached saturation at a low concentration, and exhibited a binding activity weaker than that of the chimeric antibody. The neutralizing activity against the antigen of "g-b" was significantly weak as compared to that of the chimeric antibody.

(xi) Combination of the humanized H chain versions "b1" and "d1", and the humanized L chain version "b"

Antibodies ("b1-b" and "d1-b", respectively) which are the humanized H chain versions "b1" and "d1" combined with the humanized L chain version "b" were generated. Almost no antibody was expressed in any of them. For these, antibodies combined with a chimeric L chain were generated, but were not expressed.

(xii) Combination of the humanized H chain versions "b3" and "d3", and the humanized L chain version "b"

Antibodies ("b3-b" and "d3-b", respectively) which are the humanized H chain versions "b3" and "d3" combined with the humanized L chain version "b" were generated. The binding activity to the antigen of "d3-b" was slightly lower than that of the chimeric antibody, and that of "b3-b" was much lower. The neutralizing activity against the antigen of "b3-b" was higher than that of "b-b", but was lower than that of the chimeric antibody, and "d3-b" and "b-b" remained equal in activity.

(xiii) Combination of the humanized H chain versions "i" and "j", and a chimeric L chain and the humanized L chain version "b"

Antibodies ("i-ch" and "j-ch", respectively) which are the humanized H chain versions "i" and "j" combined with a chimeric L chain, and antibodies ("i-b" and "j-b", respectively) combined with the humanized L chain version "b" were generated, and were tested for the binding

activity to the antigen and the neutralizing activity against the antigen. The binding activity of any of the antibodies was almost equal to that of the chimeric antibody. "i-ch" exhibited the neutralizing activity higher than that of the chimeric antibody, and "j-ch" was significantly lower than that of the chimeric antibody. "i-b" exhibited the neutralizing activity equal to that of the chimeric antibody, and "j-b" exhibited a significantly weaker neutralizing activity than that of that of the chimeric antibody.

(xiv) The humanized L chain versions "b1" and "b2"

When antibodies ("ch-b1" and "ch-b2", respectively) which are the humanized L chain versions "b1" and "b2" combined with a chimeric H chain were generated, both of them exhibited the binding activity to the antigen equal to that of the chimeric antibody. For the neutralizing activity against the antigen, "ch-b1" exhibited the binding activity equal to that of the chimeric antibody, while "ch-b2" exhibited an activity slightly higher than that of the chimeric antibody at the high concentration. Versions "b1" and "b2" can be candidates of a humanized antibody L chain, but "b2" is superior in that it has a stronger activity.

(xv) Combination of the humanized H chain version "b" and the humanized L chain version "b2"

An antibody ("b-b2") which is the humanized H chain version "b" combined with the humanized L chain version "b2" was generated, and was tested for the binding activity to the antigen and the neutralizing activity against the antigen. The binding activity was slightly lower than that of the chimeric antibody. The neutralizing activity, though slightly higher than that of "b-b", was lower than that of "i-b".

(xvi) Combination of the humanized H chain version "i" and, the humanized L chain version "b1" or "b2"

Antibodies ("i-b1" and "i-b2", respectively) which

are the humanized H chain version "i" combined with the humanized L chain version "b1" or "b2" were generated, and were tested for the binding activity to the antigen and the neutralizing activity against the antigen. The binding activity of "i-b2" was almost equal to that of the chimeric antibody, and that of "i-b1" was slightly lower than that of chimeric antibody. The neutralizing activity of "i-b1" and "i-b2" was higher than that of the chimeric antibody and "i-b", which was in a decreasing order of "i-b2" > "i-b1".

Reference Example 7. Preparation of CHO cell-producing humanized antibody and the evaluation of its activity

(1) Establishment of a cell line that stably produces CHO

In order to establish cell lines that stably produce a humanized antibody (b-b, i-b, and i-b2), an antibody expression gene vector was introduced into CHO cells (DG44) acclaimed to a serum-free medium.

Plasmid DNA, hHvb-hLvb/N5KG4P, hHvi-hLvb/N5KG4P, and hHvi-hLvb2/N5KG4P were digested with the restriction enzyme SspI (Takara Shuzo) and linearized, which was extracted with phenol and chloroform, and purified by ethanol precipitation. The linearized expression gene vector was introduced into the DG44 cells using the electroporation instrument (Gene Pulser; Bio Rad). The DG44 cells were suspended in PBS at a cell concentration of 1×10^7 cells/ml, and to about 0.8 ml of this suspension 10 or 50 μg of the DNA was added, which was subjected to pulses of 1,500 V and 25 μF capacity.

After 10 minutes of the recovery period at room temperature, the treated cells were suspended in a CHO-S-SFMII medium (GIBCO) containing hypoxanthine/thymidine (GIBCO) (hereinafter referred to as HT), which was inoculated on two 96-well plates (Falcon) at 100 μl/well,

and cultured in a CO_2 incubator. Eight to nine hours after the start of culturing, 100 μl/well of the CHO-S-SFMII medium containing HT and 1 mg/ml GENETICIN (GIBCO) was added to change to 500 μg/ml of the GENETICIN selection medium, and the cells into which the antibody gene had been introduced were selected. The medium was changed with a fresh one once every 3-4 days with 1/2 the volume. At a time point about 2 weeks after changing to the selection medium, an aliquot of the culture supernatant was recovered from the well in which a favorable cell growth was observed 4-5 days later. The concentration of antibody expressed in the culture supernatant was measured by the ELISA described above for measuring antibody concentration, and cells having a high production yield of antibody were selected.

(2) Large scale purification of humanized antibody

After the DG44 cell lines selected as above that produce the humanized antibody ("b-b", "i-b", and "i-b2") were cultured for a few days in a 500 ml/bottle of the CHO-S-SFMII medium using a 2 L roller bottle (CORNING), the culture medium was harvested and a fresh CHO-S-SFMII medium was added and cultured again. The culture medium was centrifuged to remove the cell debris, and filtered with a 0.22 μm or 0.45 μm filter. By repeating this, a total of about 2 L each of the culture supernatant was obtained. From the culture supernatant obtained, antibody was purified by the ConSep LC100 system (Millipore) connected to the Protein A affinity column (Poros).

(3) Measurement of antibody concentration by ELISA

ELISA plates for measurement of antibody concentration were prepared as follows: Each well of a 96-well ELISA plate (Maxisorp, NUNC) was immobilized with 100 μl of goat anti-human IgGγ antibody (BioSource) prepared to a concentration of 1 μg/ml with CB. After

blocking with 200 μl of DB, the culture supernatant of the CHO cells in which antibody had been expressed or the purified antibody was serially diluted with DB, and added to each well.

After incubating at room temperature for 1 hour and washing with RB, 100 μl of alkaline phosphatase-conjugated goat anti-human IgGγ antibody (BioSource) diluted 1000-fold with DB was added. After incubating at room temperature for 1 hour and washing with RB, 100 μl of the substrate solution was added, and then the absorbance at 405/655 nm was measured using the Microplate Reader (Bio Rad). As the standard for the measurement of concentration, human IgG4κ (The Binding Site) was used.

(4) Measurement of activity of binding to the antigen

Cell ELISA plates for measurement of antigen binding were prepared as follows. Cells used were human bladder carcinoma cells J82 (ATCC HTB-1), which were inoculated onto a 96-well cell culture plate at a cell count of 1×10^5 cells. This was cultured (RPMI1640 medium containing 10% fetal bovine serum (GIBCO)) for one day in a CO_2 incubator to allow the cells to be attached thereto. After discarding the culture liquid, each well was washed twice with PBS. 100 μl of PFA/PBS was added to each well, and placed on ice for 10 minutes to immobilize the cells.

PFA/PBS was discarded, and each well was washed twice with 300 μl of PBS and then blocked with 250 μl of DB. Based on the above result of measurement, the purified antibody was serially diluted with DB starting at 10 μg/ml by a factor of 2, 100 μl of which was added to each well. After incubating at room temperature for 2 hours and washing with RB, 100 μl of alkaline

phosphatase-conjugated goat anti-human IgGγ antibody (BioSource) diluted 1000-fold with DB was added. After incubating at room temperature for 1 hour and washing with RB, 100 µl of the substrate solution was added, and then absorbance at 405/655 nm was measured using the Microplate Reader (Bio-Rad).

(5) Measurement of neutralizing activity against TF (Factor inhibiting activity against the FXa production)

The Factor Xa production-inhibiting activity of humanized antibody was measured with the inhibiting activity against the Factor Xa production activity by the human placenta-derived thromboplastin, Thromborel S (Boehringer AG), as an index. Thus, 60 µl of the buffer (TBS containing 5 mM $CaCl_2$ and 0.1% BSA) was added to 10 µl of 5 mg/ml Thromborel S and 10 µl of the antibody, which was then incubated in a 96-well plate at room temperature for 1 hour. The antibody was serially diluted with the buffer starting at 200 µg/ml by a factor of 5.

Ten µl each of 3.245 µg/ml human Factor X (Celsus Laboratories) and 82.5 ng/ml human Factor VIIa (Enzyme Research) were added thereto, and were further incubated at room temperature for 45 minutes. Ten µl of 0.5 M EDTA was added to stop the reaction. Fifty µl of the chromogenic substrate solution was added thereto and the absorbance at 405/655 nm was determined by the Microplate Reader (Bio Rad). After reacting at room temperature for 30 minutes, the absorbance at 405/655 nm was measured again. The residual activity (%) was determined from each change in absorbance with the absorbance change for 30 minutes at no antibody addition as a 100% activity.

The chromogenic substrate solution was prepared by dissolving the Testzyme chromogenic substrate S-2222 (Chromogenix) according to the attached instructions, and

mixing with a polybrene solution (0.6 mg/ml hexadimethylene bromide, SIGMA) at 1:1.

(6) Measurement of neutralizing activity against TF (inhibiting activity against the FX-binding)

The inhibiting activity against the FX-binding of humanized antibody was measured using the human placenta-derived thromboplastin, Thromborel S (Boehringer AG), in which a complex of TF and Factor VIIa had previously been formed and the inhibiting activity against the FX-binding was measured with the Factor Xa production activity of the TF-FVlla complex as an index. Thus, 60 μl of the buffer (TBS containing 5 mM $CaCl_2$ and 0.1% BSA) was added to 10 μl of 5 mg/ml Thromborel S and 10 μl of 82.5 ng/ml human Factor VIIa (Enzyme Research), which was preincubated in a 96-well plate at room temperature for 1 hour.

Ten μl of the antibody solution was added thereto, incubated at room temperature for 5 minutes, and 10 μl of 3.245 μg/ml human Factor X (Celsus Laboratories) was added and was further incubated at room temperature for 45 minutes. The antibody was serially diluted with the buffer starting at 200 μg/ml by a factor of 2. Ten μl of 0.5 M EDTA was added to stop the reaction. Fifty μl of the chromogenic substrate solution was added thereto and the absorbance at 405/655 nm was determined by the Microplate Reader (Bio Rad). After reacting at room temperature for 30 minutes, the absorbance at 405/655 nm was measured again. The residual activity (%) was determined from each change in absorbance with the absorbance change for 30 minutes at no antibody addition as a 100% activity.

The chromogenic substrate solution was prepared by dissolving the Testzyme chromogenic substrate S-2222 (Chromogenix) according to the attached instructions, and mixing with a polybrene solution (0.6 mg/ml

hexadimethylene bromide, SIGMA) at 1:1.

(7) Measurement of neutralizing activity against the inhibiting activity against the (plasma coagulation)

The neutralizing activity against TF (inhibiting activity against the plasma coagulation) of humanized antibody was measured using, as an index, prothrombin time determined using the human placenta-derived thromboplastin, Thromborel S (Boehringer AG). Thus, 100 µl of human plasma (Cosmo Bio) was placed into a sample cup, to which 50 µl of antibody diluted at various concentrations was added, and heated at 37°C for 3 minutes. Fifty µl of 1.25 mg/ml Thromborel S that had previously been preheated at 37°C was added to start plasma coagulation. The coagulation time was measured using the Amelung KC-10A connected to the Amelung CR-A (both from M. C. Medical).

The antibody was serially diluted with TBS containing 0.1% BSA (hereinafter referred to as BSA-TBS) starting at 80 µg/ml by a factor of 2. With the coagulation time of no antibody addition as 100% TF plasma coagulation activity, the residual TF activity was calculated from each coagulation time at antibody addition based on a standard curve obtained by plotting the concentration of Thromborel S and the coagulation time.

The standard curve was created from the various concentration of Thromborel S and the coagulation time was measured. Fifty µl of BSA-TBS was added to 50 µl of appropriately diluted Thromborel S, which was heated at 37°C for 3 minutes, 100 µl of human plasma preheated at 37°C was added to start coagulation, and the coagulation time was determined. Thromborel S was serially diluted with the Hank's buffer (GIBCO) containing 25 mM $CaCl_2$ starting at 6.25 mg/ml by a factor of 2. The Thromborel

S concentration was plotted on the abscissa, and the coagulation time on the ordinate on a log-log paper, which was rendered a standard curve.

(8) Activity evaluation

All humanized antibodies, "b-b", "i-b", and "i-b2" had an activity equal to or greater than that of the chimeric antibody (Figure 1). For inhibiting activity against FXa production, inhibiting activity FX-binding, and inhibiting activity against plasma coagulation as well, the humanized antibodies, "b-b", "i-b", and "i-b2" had an activity equal to or greater than that of the chimeric antibody, and the activity was of a decreasing order "i-b2" > "i-b" > "b-b" (Figures 2, 3, and 4).

CLAIMS

1. An experimental animal having implanted therein an animal cell to which the gene encoding human tissue factor (TF) or part thereof has been inserted and which is capable of expressing said gene, said animal being a non-human animal in which a hypercoagulable state persists for a long period of time.

2. The animal according to claim 1 wherein said part of human tissue factor lacks the intracellular region.

3. The animal according to claim 1 or 2 wherein said animal cell is a mammalian cell.

4. The animal according to claim 3 wherein said mammalian cell is a human myeloma cell.

5. The animal according to any one of claims 1 to 4 wherein said animal is a mouse.

6. The animal according to any of claims 1 to 5 wherein said hypercoagulable state is indicated by at least one of the phenomena comprising an increase in the plasma concentration of human tissue factor, a decrease in platelets, a decrease in fibrinogen, an increase in the concentration of soluble fibrin monomer complex, and an increase in the concentration of thrombin-antithrombin III complex.

7. A method of generating the animal according to any one of claims 1 to 6, wherein an animal cell to which the gene encoding human tissue factor (TF) or part thereof has been inserted and which is capable of expressing said gene is implanted to non-human animals and then an animal having a persistent hypercoagulable state is selected.

8. A method of screening an anti-thrombotic agent which method comprises using the animal according to any one of claims 1 to 6.

9. A preventive or therapeutic agent for diseases having a persistent hypercoagulable state, said agent comprising an antibody to human tissue factor (human TF).

10. The preventive or therapeutic agent according to claim 9 wherein said antibody is a polyclonal antibody.

11. The preventive or therapeutic agent according to claim 9 wherein said antibody is a monoclonal antibody.

12. The preventive or therapeutic agent according to claim 9 or 11 wherein said antibody is a recombinant antibody.

13. The preventive or therapeutic agent according to claim 9 or 12 wherein said antibody is an altered antibody.

14. The preventive or therapeutic agent according to claim 9, 12, or 13 wherein said altered antibody is a chimeric antibody or a humanized antibody.

15. The preventive or therapeutic agent according to claim 14 wherein said humanized antibody is a humanized antibody of version b-b, i-b, or i-b2.

16. The preventive or therapeutic agent according to claim 9 or any one of claims 12-15 wherein said antibody is a modified antibody.

17. The preventive or therapeutic agent according to claim 16 wherein said modified antibody is an antibody fragment Fab, $F(ab')_2$, or Fv, or a single chain Fv (scFv).

18. A preventive or therapeutic agent for a hypercoagulable state resulting from infections, said agent comprising an antibody against human tissue factor (human TF).

19. The preventive or therapeutic agent according to claim 18 wherein said antibody is a polyclonal antibody.

20. The preventive or therapeutic agent according to claim 18 wherein said antibody is a monoclonal antibody.

21. The preventive or therapeutic agent according to claim 18 or 20 wherein said antibody is a recombinant

antibody.

22. The preventive or therapeutic agent according to claim 18 or 21 wherein said antibody is an altered antibody.

23. The preventive or therapeutic agent according to claim 18, 21, or 22 wherein said altered antibody is a chimeric antibody or a humanized antibody.

24. The preventive or therapeutic agent according to claim 23 wherein said humanized antibody is a humanized antibody of version b-b, i-b, or i-b2.

25. The preventive or therapeutic agent according to claim 18 or any one of claims 21-24 wherein said antibody is a modified antibody.

26. The preventive or therapeutic agent according to claim 25 wherein said modified antibody is an antibody fragment Fab, $F(ab')_2$, or Fv, or a single chain Fv (scFv).

27. A preventive or therapeutic agent for venous thrombosis, said agent comprising an antibody to human tissue factor (human TF).

28. The preventive or therapeutic agent according to claim 27 wherein said antibody is a polyclonal antibody.

29. The preventive or therapeutic agent according to claim 27 wherein said antibody is a monoclonal antibody.

30. The preventive or therapeutic agent according to claim 27 or 29 wherein said antibody is a recombinant antibody.

31. The preventive or therapeutic agent according to claim 27 or 30 wherein said antibody is an altered antibody.

32. The preventive or therapeutic agent according to claim 27, 30, or 31 wherein said altered antibody is a chimeric antibody or a humanized antibody.

33. The preventive or therapeutic agent according to claim 32 wherein said humanized antibody is a

humanized antibody of version b-b, i-b, or i-b2.

34. The preventive or therapeutic agent according to claim 27 or any one of claims 30-33 wherein said antibody is a modified antibody.

35. The preventive or therapeutic agent according to claim 34 wherein said modified antibody is an antibody fragment Fab, $F(ab')_2$, or Fv, or a single chain Fv (scFv).

36. A preventive or therapeutic agent for arterial thrombosis, said agent comprising an antibody to human tissue factor (human TF).

37. The preventive or therapeutic agent according to claim 36 wherein said antibody is a polyclonal antibody.

38. The preventive or therapeutic agent according to claim 36 wherein said antibody is a monoclonal antibody.

39. The preventive or therapeutic agent according to claim 36 or 38 wherein said antibody is a recombinant antibody.

40. The preventive or therapeutic agent according to claim 36 or 39 wherein said antibody is an altered antibody.

41. The preventive or therapeutic agent according to claim 36, 39, or 40 wherein said altered antibody is a chimeric antibody or a humanized antibody.

42. The preventive or therapeutic agent according to claim 41 wherein said humanized antibody is a humanized antibody of version b-b, i-b, or i-b2.

43. The preventive or therapeutic agent according to claim 36 or any one of claims 39-42 wherein said antibody is a modified antibody.

44. The preventive or therapeutic agent according to claim 43 wherein said modified antibody is an antibody fragment Fab, $F(ab')_2$, or Fv, or a single chain Fv (scFv).

45. A preventive or therapeutic agent for diseases

resulting from the medial thickening of the vessels, said agent comprising an antibody to human tissue factor (human TF).

46. The preventive or therapeutic agent according to claim 45 wherein said antibody is a polyclonal antibody.

47. The preventive or therapeutic agent according to claim 45 wherein said antibody is a monoclonal antibody.

48. The preventive or therapeutic agent according to claim 45 or 47 wherein said antibody is a recombinant antibody.

49. The preventive or therapeutic agent according to claim 45 or 48 wherein said antibody is an altered antibody.

50. The preventive or therapeutic agent according to claim 45, 48, or 49 wherein said altered antibody is a chimeric antibody or a humanized antibody.

51. The preventive or therapeutic agent according to claim 50 wherein said humanized antibody is a humanized antibody of version b-b, i-b, or i-b2.

52. The preventive or therapeutic agent according to claim 43 or any one of claims 48-51 wherein said antibody is a modified antibody.

53. The preventive or therapeutic agent according to claim 52 wherein said modified antibody is an antibody fragment Fab, $F(ab')_2$, or Fv, or a single chain Fv (scFv).

ABSTRACT

This invention provides an animal having a persistent hypercoagulable state by implanting a cell, for example a tumor cell, in which the gene of human tissue factor is implanted to an experimental animal such as a mouse and then by growing said cell, thereby persistently supplying human tissue factor to said experimental animal. This animal model is useful for research and development of therapeutic agents for diseases having a persistent hypercoagulable state.

The present invention also provides preventive or therapeutic agents for diseases having a persistent hypercoagulable state, a hypercoagulable state resulting from infections, venous thrombosis, arterial thrombosis, and diseases resulting from the hypertrophy of vascular media, said agent comprising an antibody against human tissue factor (human TF) as an active ingredient.

Fig.1

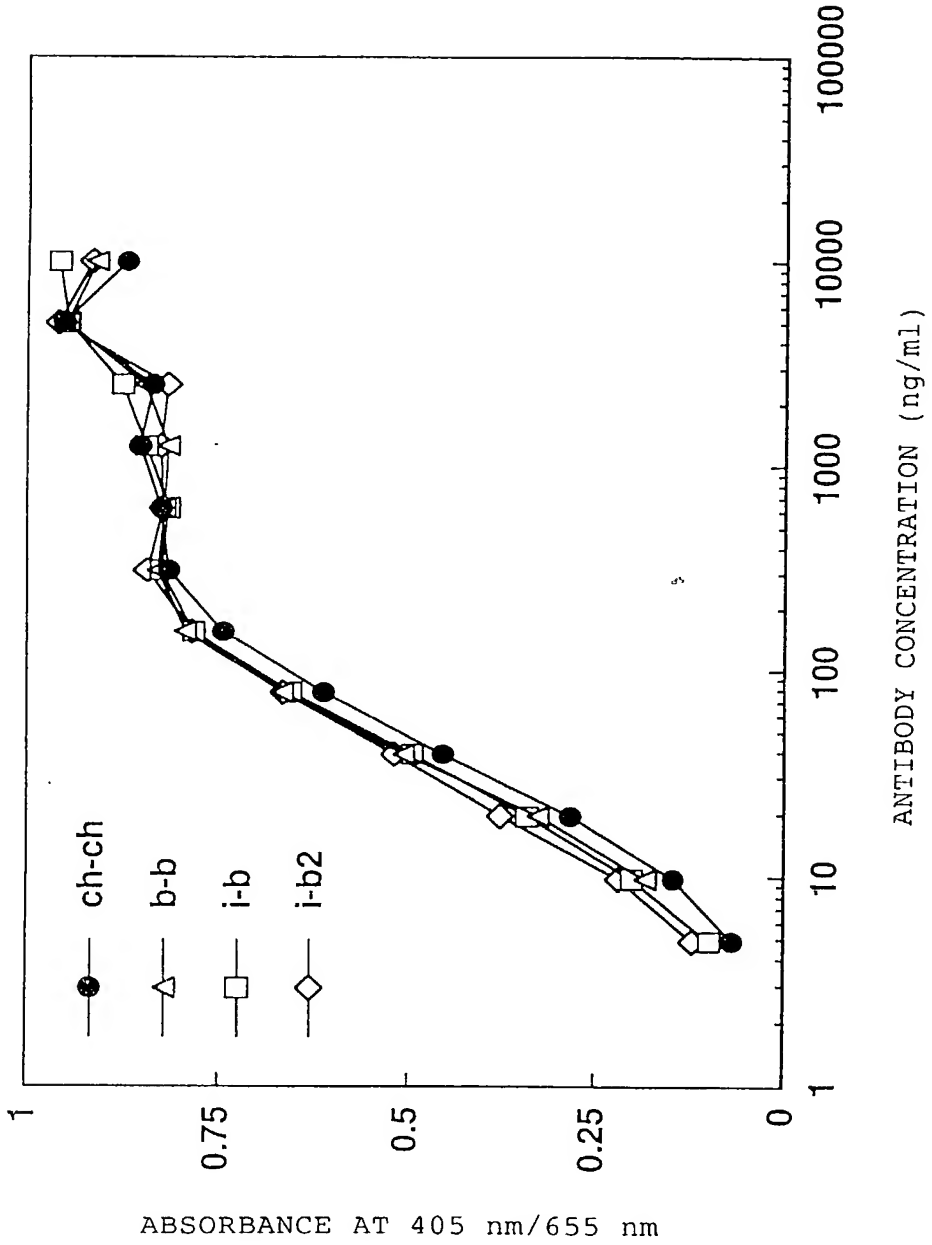
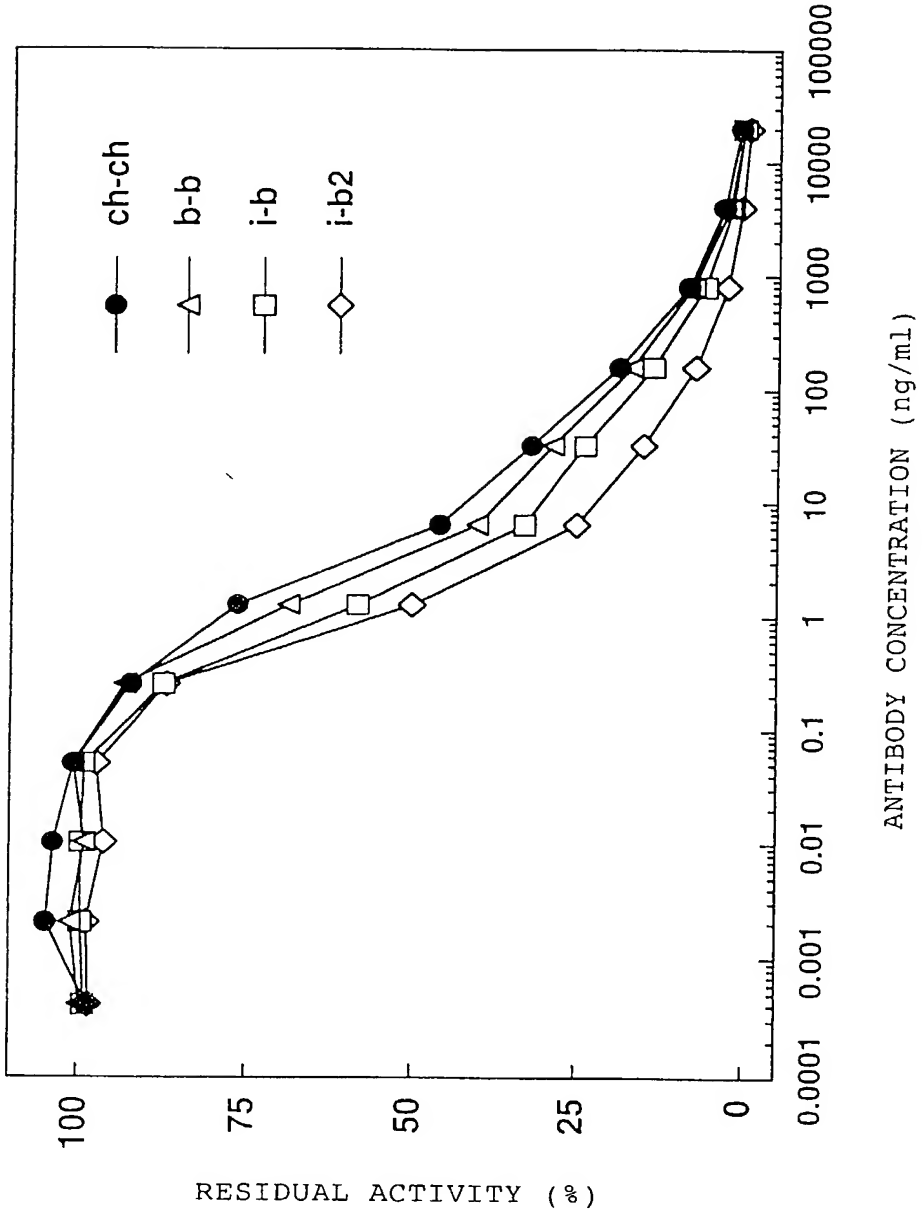

ch-ch
b-b
i-b
i-b2
1
0.75
0.5
0.25
0
ABSORBANCE AT 405 nm/655 nm
1
10
100
1000
10000
100000
ANTIBODY CONCENTRATION (ng/ml)

Fig. 2



Title: PREVENTION AND TREATMENT OF BLOOD COAGULATION-RELATED DISEASES
Inventor(s): Hiroyuki SAITO et al.
DOCKET NO.: 053466-0325

Fig. 3

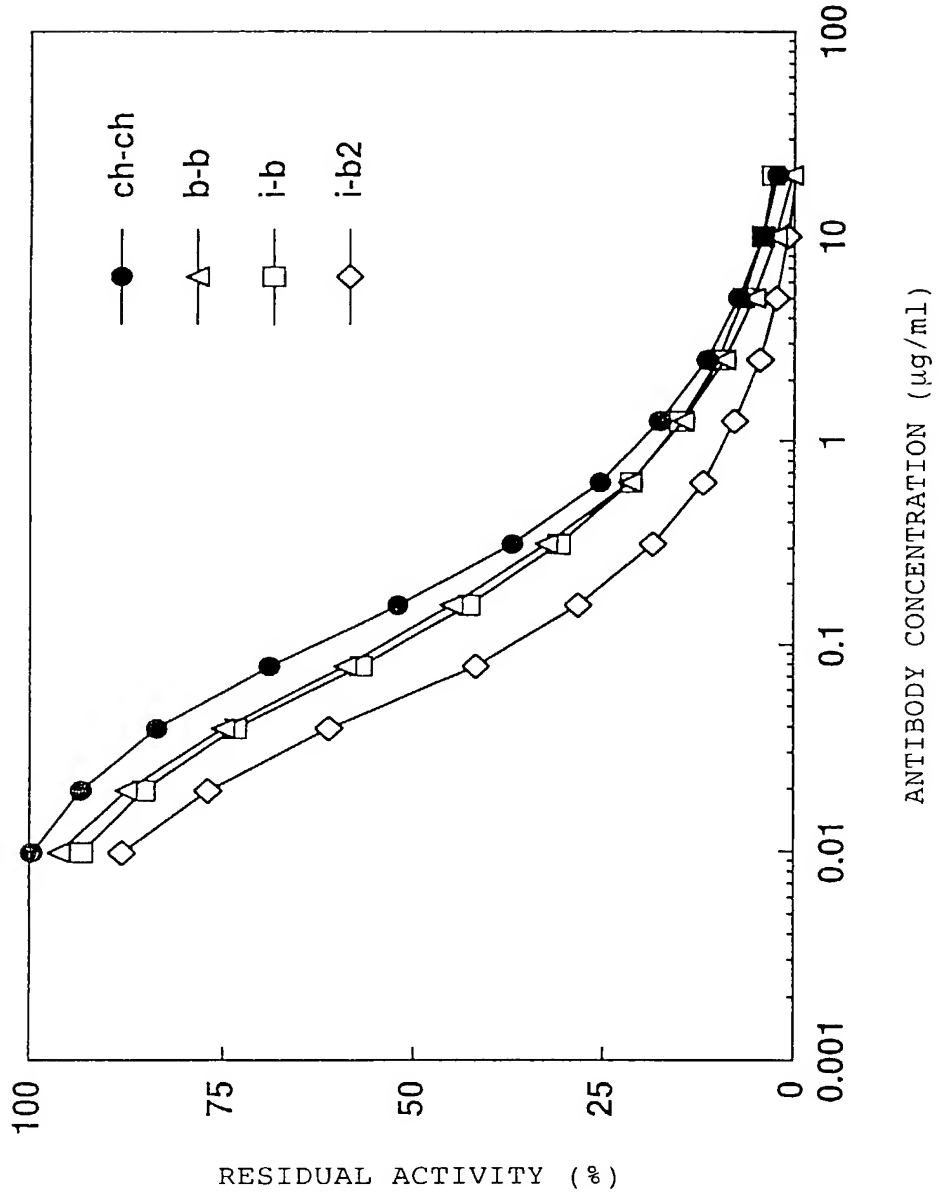


Fig. 4

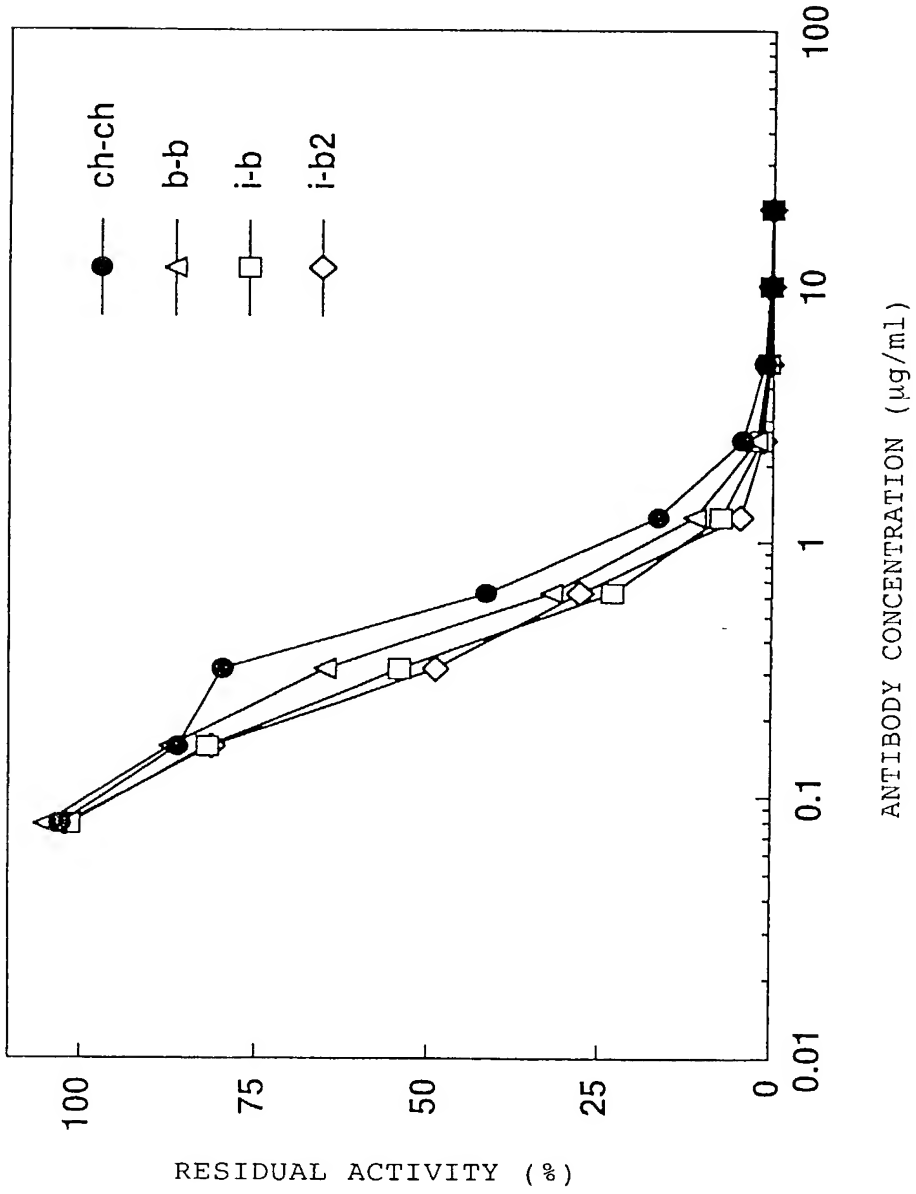


Fig. 5

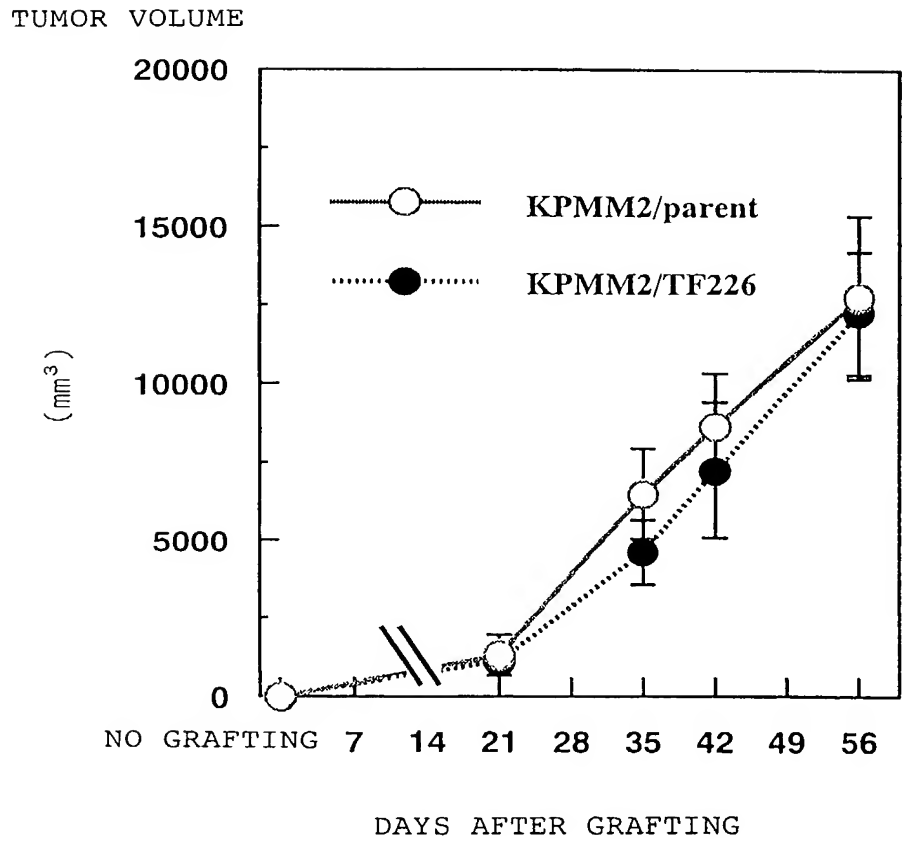


Fig. 6

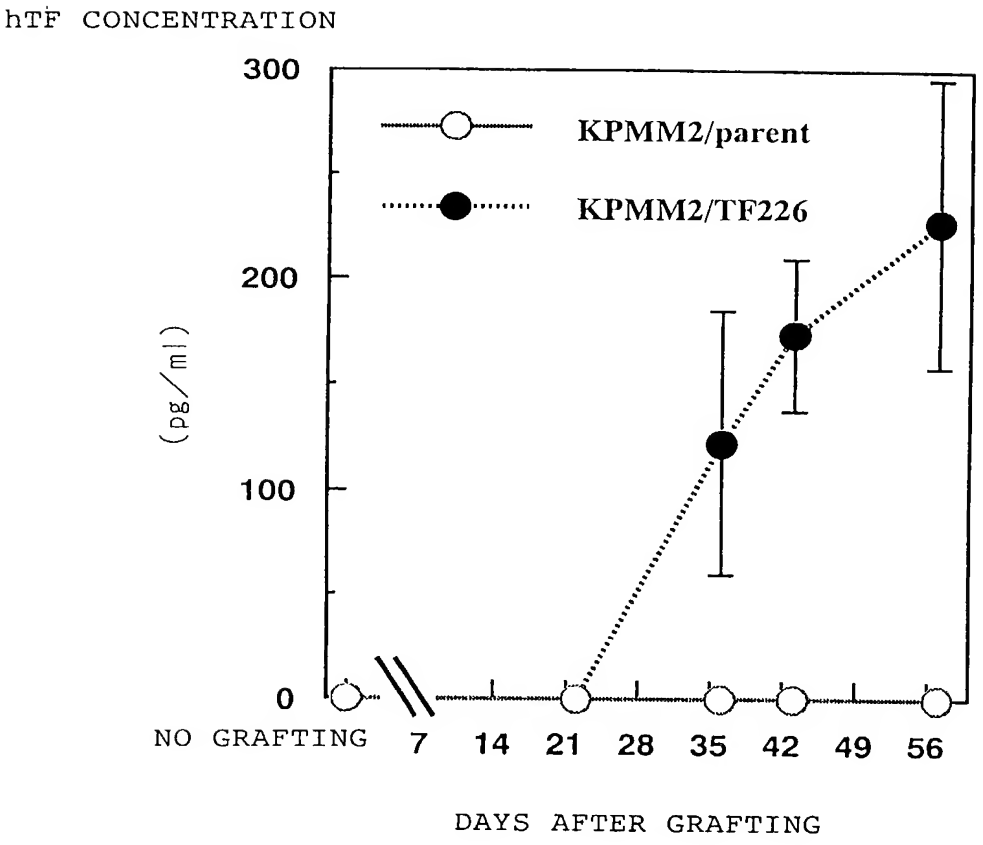


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Fig.7

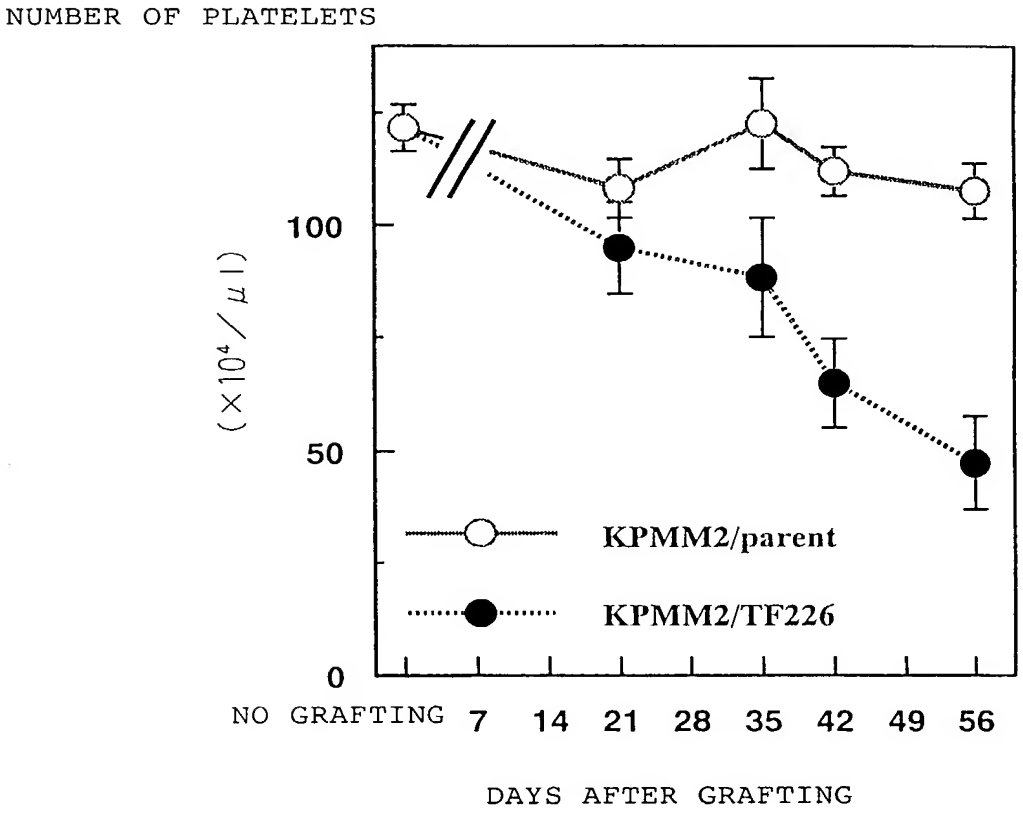

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Fig. 8

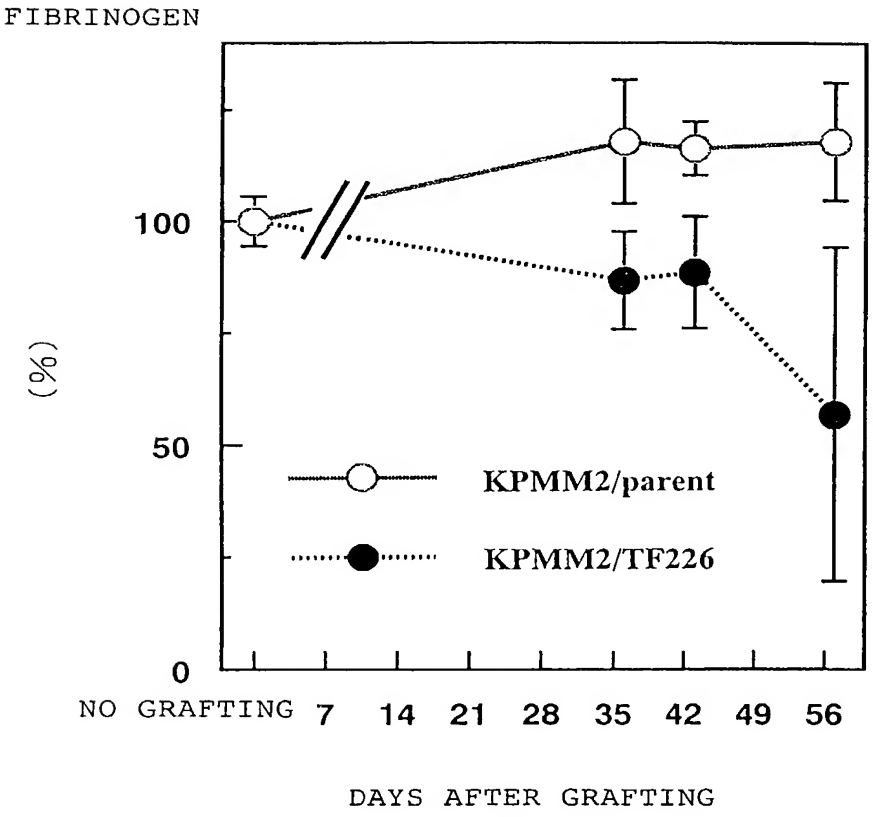
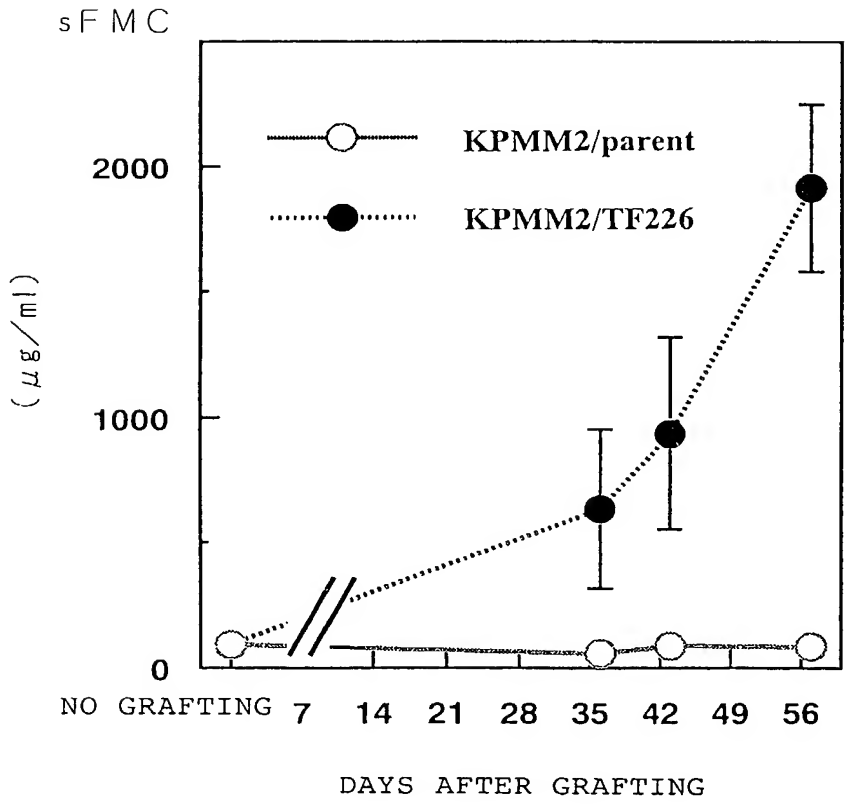


Fig.9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(μg/ml)
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1000
0
KPMM2/parent
KPMM2/TF226
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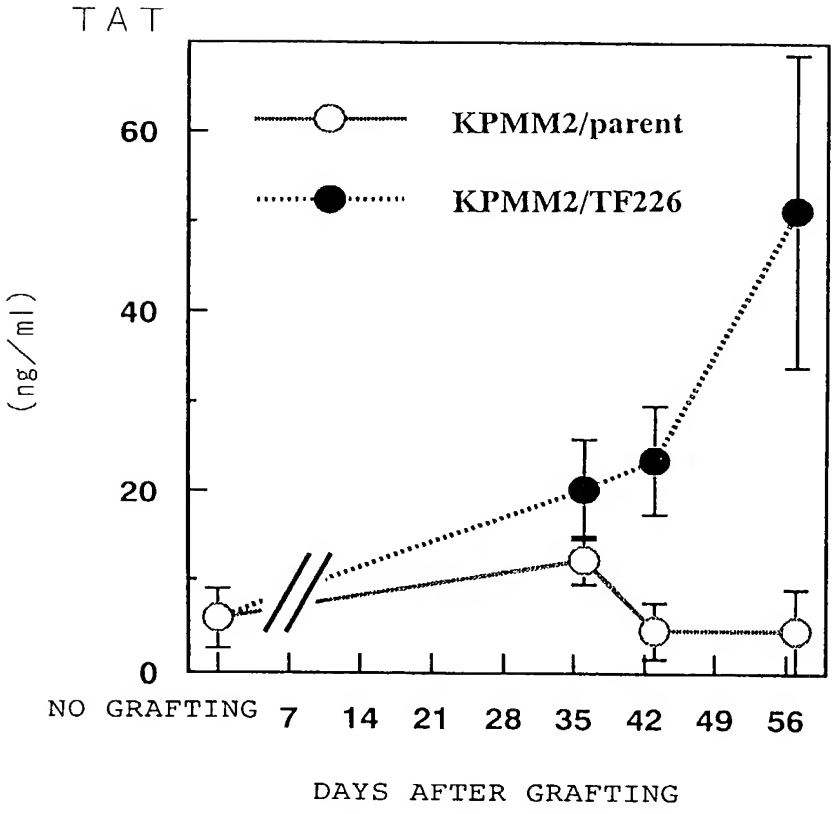
14
21
28
35
42
49
56
DAYS AFTER GRAFTING

Fig.10


TAT
(ng/ml)
60
40
20
0
KPMM2/parent
KPMM2/TF226
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7
14
21
28
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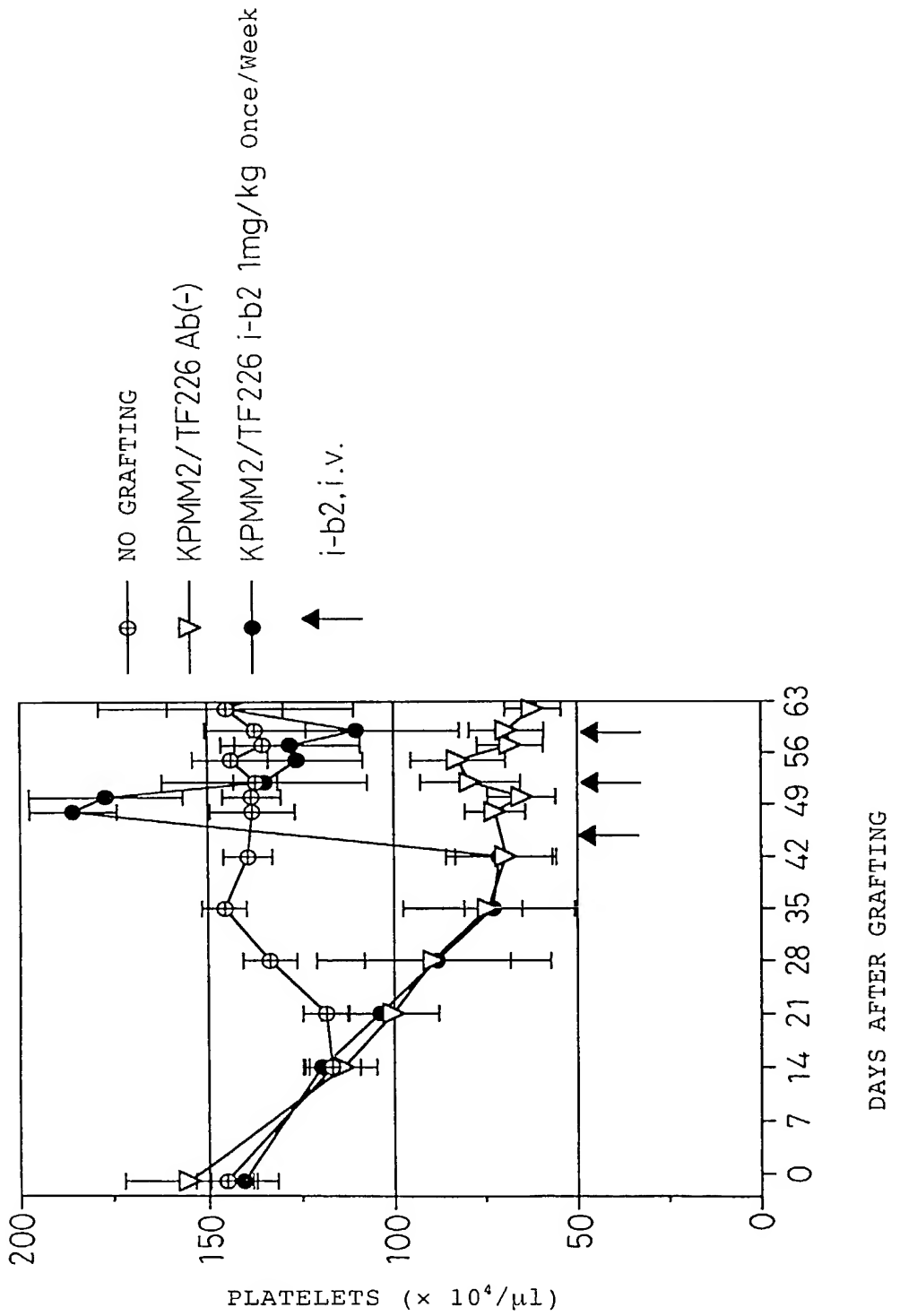
49
56
DAYS AFTER GRAFTING

Fig.11


NO GRAFTING
KPMM2/TF226 Ab(-)
KPMM2/TF226 i-b2 1mg/kg once/Week
i-b2, i.v.
PLATELETS (× 10⁴/μl)
200
150
100
50
0
0 7 14 21 28 35 42 49 56 63
DAYS AFTER GRAFTING

Title: PREVENTION AND TREATMENT OF BLOOD COAGULATION-RELATED DISEASES
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DOCKET NO.: 053466-0325

Fig.12

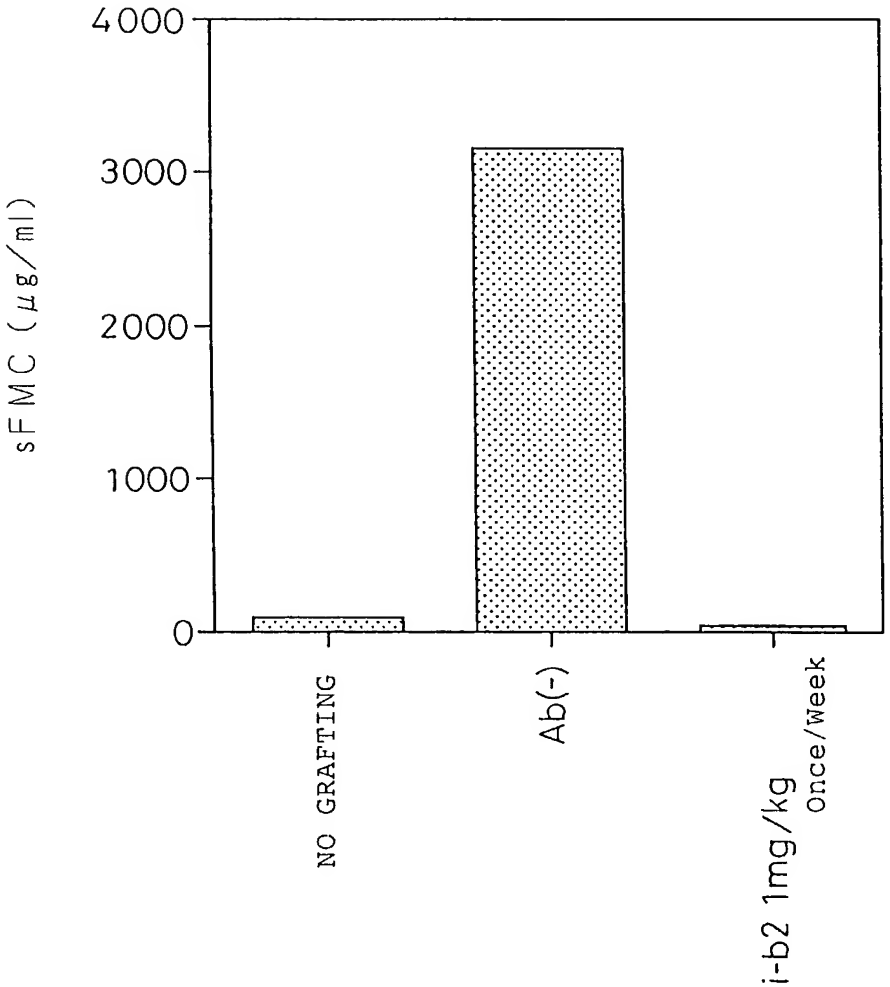


Fig.13

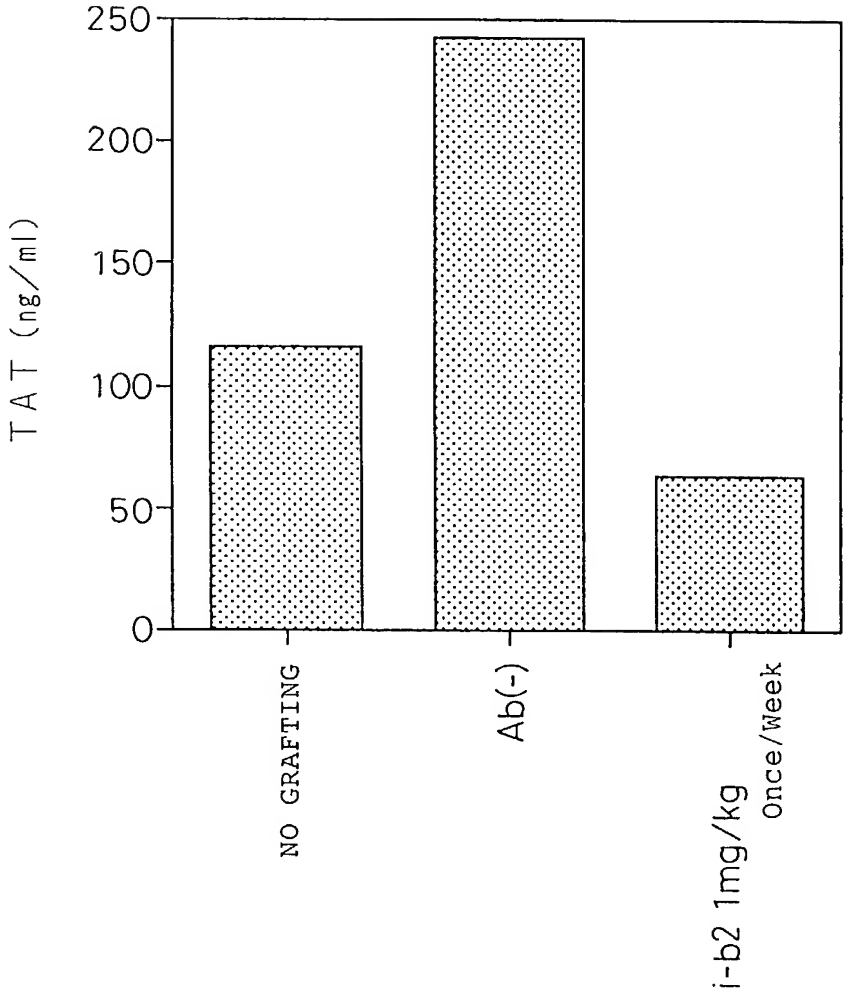
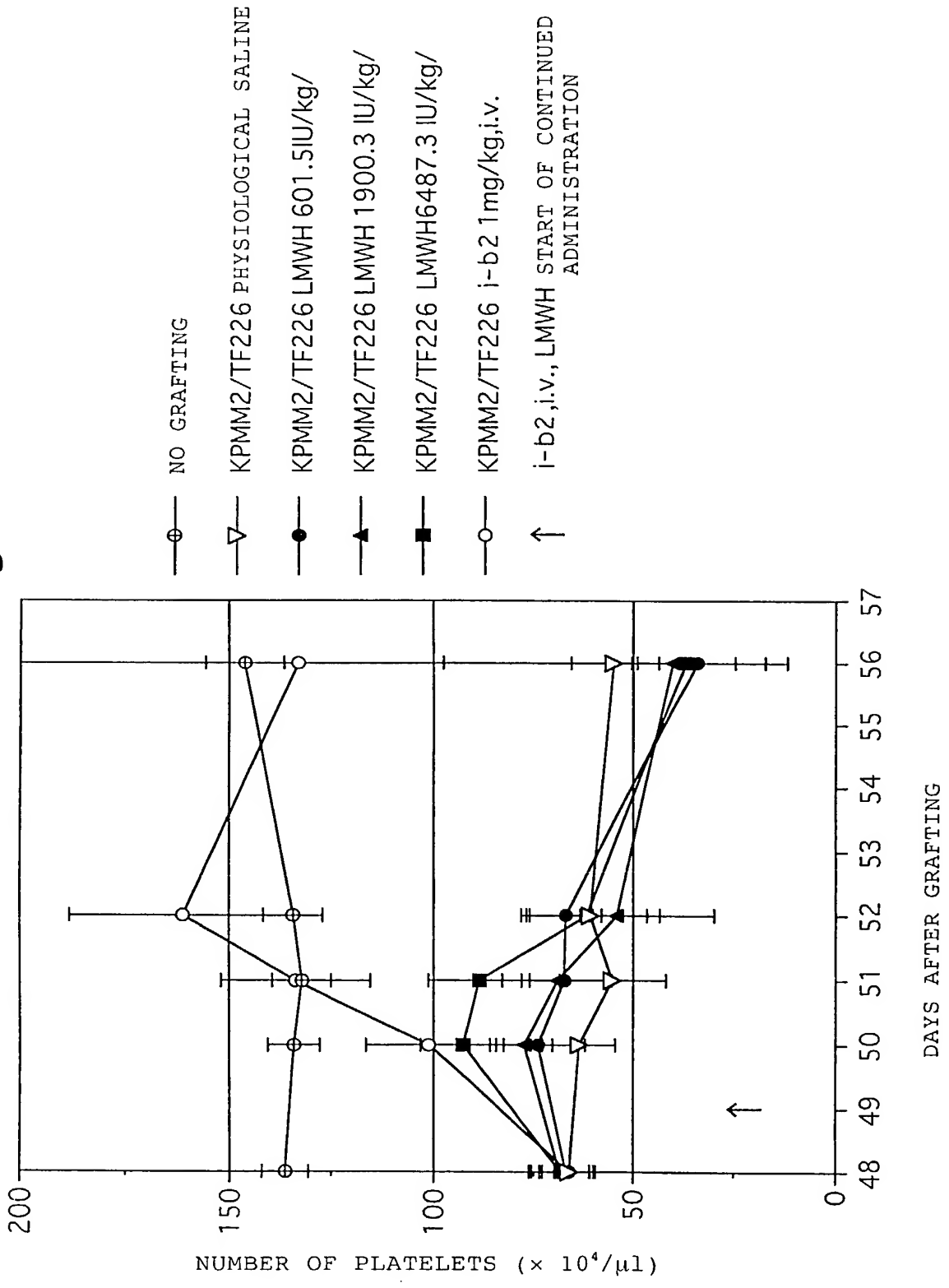


Fig.14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KPMM2/TF226 LMWH 601.5IU/kg/
KPMM2/TF226 LMWH 1900.3 IU/kg/
KPMM2/TF226 LMWH6487.3 IU/kg/
KPMM2/TF226 i-b2 1mg/kg,i.v.
i-b2,i.v., LMWH START OF CONTINUED ADMINISTRATION
NUMBER OF PLATELETS (× 10⁴/μl)
200
150
100
50
0
48
49
50
51
52
53
54
55
56
57
DAYS AFTER GRAFTING

10089501 .042202 (Foley)

PTO/SB/106(8-96)
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Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
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Declaration and Power of Attorney For Patent Application

特許出願宣言書及び委任状

Japanese Language Declaration

日本語宣言書

下記の氏名の発明者として、私は以下の通り宣言します。	As a below named inventor, I hereby declare that:
私の住所、私書箱、国籍は下記の私の氏名の後に記載された通りです。	My residence, post office address and citizenship are as stated next to my name.
下記の名称の発明に関して請求範囲に記載され、特許出願している発明内容について、私が最初かつ唯一の発明者（下記の氏名が一つの場合）もしくは最初かつ共同発明者であると（下記の名称が複数の場合）信じています。	I believe I am the original, first and sole inventor (if only one mane is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled
______	PREVENTION AND TREATMENT OF BLOOD
______	COAGULATION-RELATED DISEASES
上記発明の明細書（下記の欄でｘ印がついていない場合は、本書に添付）は、	the specification of which is attached hereto unless the following box is checked:
☐ __月__日に提出され、米国出願番号または特許協定条約国際出願番号を________とし、（該当する場合）________に訂正されました。	☒ was filed on September 29, 2000 as United States Application Number or PCT International Application Number PCT/JP00/06802 and was amended on ________ (if applicable).
私は、特許請求範囲を含む上記訂正後の明細書を検討し、内容を理解していることをここに表明します。	I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.
私は、連邦規則法典第３７編第１条５６項に定義されるとおり、特許資格の有無について重要な情報を開示する義務があることを認めます。	I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56.

Japanese Language Declaration
（日本語宣言書）

私は、米国法典第３５編１１９条(a)-(d)項又は３６５条(b)項に基き下記の、米国以外の国の少なくとも一カ国を指定している特許協力条約３６５(a)項に基づく国際出願、又は外国での特許出願もしくは発明者証の出願について外国優先権をここに主張するとともに、優先権を主張している、本出願の前に出願された特許または発明者証の外国出願を以下に、枠内をマークすることで、示しています。

I hereby claim foreign priority under Title 35, United States Code, Section 119(a)-(d) or 365(b) of any foreign application(s) for patent or inventor's certificate, or 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or PCT International application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s)
外国での先行出願

(Number) (番号)	(Country) (国名)	(Day/Month/Year Filed) (出願年月日)	Priority Claimed 優先権主張 Yes はい	No いいえ
11-281843(Pat.Appln.)	Japan	1/October/1999	☒	☐
11-282120(Pat.Appln.)	Japan	1/October/1999	☒	☐

* Please see the Page 3 of 5

私は、第３５編米国法典第１１９条(e)項に基いて下記の米国特許出願規定に記載された権利をここに主張いたします。

I hereby claim the benefit under Title 35, United States Code, Section 119(e) of any United States provisional application(s) listed below.

(Application No.) (出願番号)	(Filing Date) (出願日)	(Application No.) (出願番号)	(Filing Date) (出願日)

私は、下記の米国法典第３５編１２０条に基いて下記の米国特許出願に記載された権利、又は米国を指定している特許協力条約３６５条(c)に基づく権利をここに主張します。また、本出願の各請求範囲の内容が米国法典第３５編１１２条第１項又は特許協力条約で規定された方法で先行する米国特許出願に開示されていない限り、その先行米国出願書提出日以降で本出願書の日本国内または特許協力条約国際提出日までの期間中に入手された、連邦規則法典第３７編１条５６項で定義された特許資格の有無に関する重要な情報について開示義務があることを認識しています。

I hereby claim the benefit under Title 35, United States Code, Section 120 of any United States application(s), or 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of Title 35, United States Code Section 112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56 which became available between the filing date of the prior application and the national or PCT International filing date of application.

(Application No.) (出願番号)	(Filing Date) (出願日)	(Status: Patented, Pending, Abandoned) (現況：特許許可済、係属中、放棄済)

私は、私自身の知識に基づいて本宣言書中で私が行なう表明が真実であり、かつ私の入手した情報と私の信じるところに基づく表明が全て真実であると信じていること、さらに故意になされた虚偽の表明及びそれと同等の行為は米国法典第１８編第１００１条に基づき、罰金または拘禁、もしくはその両方により処罰されること、そしてそのような故意による虚偽の声明を行なえば、出願した、又は既に許可された特許の有効性が失われることを認識し、よってここに上記のごとく宣言を致します。

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Additional Prior Foreign Application(s)
(外国での先行出願)

Priority Not Claimed
優先権主張しない

(Number) (番号)	(Country) (国名)	(Day/Month/Year Filed) (出願年月日)	
11-282134 (Pat. Appln.)	Japan	1/October/1999	☐
11-282167 (Pat. Appln.)	Japan	1/October/1999	☐
11-282188 (Pat. Appln.)	Japan	1/October/1999	☐
11-282192 (Pat. Appln.)	Japan	1/October/1999	☐
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			☐
			☐

PTO/SB/106(8-96)
Approved for use through 9/30/98 OMB 0651-0032
Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

Japanese Language Declaration
(日本語宣言書)

委任状： 私は下記の発明者として、本出願に関する一切の手続きを米特許商標局に対して遂行する弁理士または代理人として、下記の者を指名いたします。（弁護士、または代理人の氏名及び登録番号を明記のこと）

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith (*list name and registration number*).

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John P. Isacson, Reg. No. 33,715
Eugene M. Lee, Reg. No. 32,039
Richard Linn, Reg. No. 25,144
Peter G. Mack, Reg. No. 26,001

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発明者の署名 日付	Inventor's signature Hiroyuki Saito Date April 1, 2002
住所	Residence Gotenba-shi, Shizuoka, Japan
国籍	Citizenship Japanese
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第二共同発明者の署名 日付	Second Inventor's signature Takehisa Kitazawa Date April 1, 2002
住所	Residence Gotenba-shi, Shizuoka, Japan
国籍	Citizenship Japanese
私書箱	Post Office Address c/o CHUGAI SEIYAKU KABUSHIKI KAISHA, 135, Komakado 1-chome, Gotenba-shi, Shizuoka 412-8513 Japan

(第三以降の共同発明者についても同様に記載し、署名をすること)

(Supply similar information and signature for third and subsequesnt joint inventors.)

Japanese Language Declaration

第三共同発明者氏名	Full name of third joint inventor Kazutaka Yoshihashi
同発明者の署名 日付	Inventor's signature Date Kazutaka Yoshihashi April 1, 2002
住所	Residence Gotenba-shi, Shizuoka, Japan
国籍	Citizenship Japanese
郵便の宛先	Post Office Address c/o CHUGAI SEIYAKU KABUSHIKI KAISHA, 135, Komakado 1-chome, Gotenba-shi, Shizuoka 412-8513 Japan
第四共同発明者	Full name of fourth joint inventor, if any Kunihiro Hattori
同発明者の署名 日付	Inventor's signature Date Kunihiro Hattori April 1, 2002
住所	Residence Gotenba-shi, Shizuoka, Japan
国籍	Citizenship Japanese
郵便の宛先	Post Office Address c/o CHUGAI SEIYAKU KABUSHIKI KAISHA, 135, Komakado 1-chome, Gotenba-shi, Shizuoka 412-8513 Japan

第五共同発明者氏名	Full name of fifth joint inventor
同発明者の署名 日付	Inventor's signature Date
住所	Residence
国籍	Citizenship
郵便の宛先	Post Office Address
第六共同発明者	Full name of sixth joint inventor, if any
同発明者の署名 日付	Inventor's signature Date
住所	Residence
国籍	Citizenship
郵便の宛先	Post Office Address

SEQUENCE LISTING

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gtc aat tca gag gtt cag ctg cag cag tct ggg act aac ctt gtg agg    96
Val Asn Ser Glu Val Gln Leu Gln Gln Ser Gly Thr Asn Leu Val Arg
            1               5                  10

cca ggg gcc tta gtc aag ttg tcc tgc aaa ggt tct ggc ttc aac att   144
Pro Gly Ala Leu Val Lys Leu Ser Cys Lys Gly Ser Gly Phe Asn Ile
     15                  20                  25

aaa gac tac tat atg cac tgg gtg aag cag agg cct gaa cag ggc ctg   192
Lys Asp Tyr Tyr Met His Trp Val Lys Gln Arg Pro Glu Gln Gly Leu
 30                  35                  40                  45

gag tgg att gga ggg aat gat cct gcg aat ggt cat agt atg tat gac   240
Glu Trp Ile Gly Gly Asn Asp Pro Ala Asn Gly His Ser Met Tyr Asp
                50                  55                  60

ccg aaa ttc cag ggc aag gcc agt ata aca gca gac aca tcc tcc aac   288
Pro Lys Phe Gln Gly Lys Ala Ser Ile Thr Ala Asp Thr Ser Ser Asn
            65                  70                  75
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Thr Ala Tyr Leu Gln Leu Ser Ser Leu Thr Ser Glu Asp Thr Ala Val
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tat ttc tgt gct aga gac tcg ggc tat gct atg gac tac tgg ggt caa 384
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gca tcg ctg gga gag aga gtc act atc act tgc aag gcg agt cag gac 144
Ala Ser Leu Gly Glu Arg Val Thr Ile Thr Cys Lys Ala Ser Gln Asp
15 20 25
att aaa agc ttt tta agt tgg tac cag caa aaa cca tgg aaa tct cct 192
Ile Lys Ser Phe Leu Ser Trp Tyr Gln Gln Lys Pro Trp Lys Ser Pro
30 35 40

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Lys Thr Leu Ile Tyr Tyr Ala Thr Ser Leu Ala Asp Gly Val Pro Ser
45 50 55 60
aga ttc agt ggc agt gga tct ggg caa gat tat tct cta acc atc aac 288
Arg Phe Ser Gly Ser Gly Ser Gly Gln Asp Tyr Ser Leu Thr Ile Asn
65 70 75
aac ctg gag tct gac gat aca gca act tat tat tgt cta cag cat ggt 336
Asn Leu Glu Ser Asp Asp Thr Ala Thr Tyr Tyr Cys Leu Gln His Gly
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gag agc ccg tac acg ttc gga ggg ggg acc aag ctg gaa ata aaa 381
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Met Lys Cys Ser Trp Val Ile Phe Phe Leu Met Ala Val Val Thr Gly
                -15                 -10                 -5

gtt aac tca cag gtg cag ctg ttg gag tct gga gct gtg ctg gca agg    96
Val Asn Ser Gln Val Gln Leu Leu Glu Ser Gly Ala Val Leu Ala Arg
            1               5                  10

cct ggg act tcc gtg aag atc tcc tgc aag gct tcc gga ttc aac att   144
Pro Gly Thr Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Phe Asn Ile
     15                  20                  25

aaa gac tac tat atg cat tgg gta aaa cag agg cct gga cag ggt cta   192
Lys Asp Tyr Tyr Met His Trp Val Lys Gln Arg Pro Gly Gln Gly Leu
 30                  35                  40                  45

gaa tgg att ggt ggg aat gat cct gcg aat ggc cat agt atg tat gac   240
Glu Trp Ile Gly Gly Asn Asp Pro Ala Asn Gly His Ser Met Tyr Asp
                50                  55                  60

ccg aaa ttc cag ggc agg gcc aaa ctg act gca gcc aca tcc gcc agt   288
Pro Lys Phe Gln Gly Arg Ala Lys Leu Thr Ala Ala Thr Ser Ala Ser
            65                  70                  75

att gcc tac ttg gag ttc tcg agc ctg aca aat gag gat tct gcg gtc   336
Ile Ala Tyr Leu Glu Phe Ser Ser Leu Thr Asn Glu Asp Ser Ala Val
        80                  85                  90

tat tac tgt gca aga gac tcg ggc tat gcc atg gac tac tgg ggc caa   384
Tyr Tyr Cys Ala Arg Asp Ser Gly Tyr Ala Met Asp Tyr Trp Gly Gln
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20 25 30
Tyr Met His Trp Val Lys Gln Arg Pro Gly Gln Gly Leu Glu Trp Ile
35 40 45
Gly Gly Asn Asp Pro Ala Asn Gly His Ser Met Tyr Asp Pro Lys Phe
50 55 60
Gln Gly Arg Ala Lys Leu Thr Ala Ala Thr Ser Ala Ser Ile Ala Tyr
65 70 75 80
Leu Glu Phe Ser Ser Leu Thr Asn Glu Asp Ser Ala Val Tyr Tyr Cys
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<223> FR Shuffling primer F3NMBS

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gtt aac tca cag gtg cag ctg ttg gag tct gga gct gtg ctg gca agg    96
Val Asn Ser Gln Val Gln Leu Leu Glu Ser Gly Ala Val Leu Ala Arg
             1               5                  10
cct ggg act tcc gtg aag atc tcc tgc aag gct tcc gga ttc aac att   144
Pro Gly Thr Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Phe Asn Ile
     15                  20                  25
aaa gac tac tat atg cat tgg gta aaa cag agg cct gga cag ggt cta   192
Lys Asp Tyr Tyr Met His Trp Val Lys Gln Arg Pro Gly Gln Gly Leu
 30                  35                  40                  45
gaa tgg att ggt ggg aat gat cct gcg aat ggc cat agt atg tat gac   240
Glu Trp Ile Gly Gly Asn Asp Pro Ala Asn Gly His Ser Met Tyr Asp
                50                  55                  60
ccg aaa ttc cag ggc cga gtc aca atc act gca gac aca tcc acg aac   288
Pro Lys Phe Gln Gly Arg Val Thr Ile Thr Ala Asp Thr Ser Thr Asn
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aca gcc tac atg gag ctc tcg agt ctg aga tct gag gac aca gcc att   336
Thr Ala Tyr Met Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Ile
         80                  85                  90
tat tac tgt gca aga gac tcg ggc tat gcc atg gac tac tgg ggc caa   384
Tyr Tyr Cys Ala Arg Asp Ser Gly Tyr Ala Met Asp Tyr Trp Gly Gln
     95                  100                 105
ggc acc ctg gtc acc gtc tcc tca gct agc                          414
Gly Thr Leu Val Thr Val Ser Ser Ala Ser
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<223> Amino acid sequence of version "b" of humanized H chain V region
<400> 29

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             20                  25                  30
Tyr Met His Trp Val Lys Gln Arg Pro Gly Gln Gly Leu Glu Trp Ile
         35                  40                  45
Gly Gly Asn Asp Pro Ala Asn Gly His Ser Met Tyr Asp Pro Lys Phe
     50                  55                  60
Gln Gly Arg Val Thr Ile Thr Ala Asp Thr Ser Thr Asn Thr Ala Tyr
 65                  70                  75                  80
Met Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Ile Tyr Tyr Cys
                 85                  90                  95
Ala Arg Asp Ser Gly Tyr Ala Met Asp Tyr Trp Gly Gln Gly Thr Leu
            100                 105                 110
Val Thr Val Ser Ser Ala Ser
        115
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               -15                 -10                  -5
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gtt aac tca cag gtg cag ctg ttg gag tct gga gct gtg ctg gca agg 96
Val Asn Ser Gln Val Gln Leu Leu Glu Ser Gly Ala Val Leu Ala Arg
1 5 10
cct ggg act tcc gtg aag atc tcc tgc aag gct tcc gga ttc aac att 144
Pro Gly Thr Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Phe Asn Ile
15 20 25
aaa gac tac tat atg cat tgg gta aaa cag agg cct gga cag ggt cta 192
Lys Asp Tyr Tyr Met His Trp Val Lys Gln Arg Pro Gly Gln Gly Leu
30 35 40 45
gaa tgg att ggt ggg aat gat cct gcg aat ggc cat agt atg tat gac 240
Glu Trp Ile Gly Gly Asn Asp Pro Ala Asn Gly His Ser Met Tyr Asp
50 55 60
ccg aaa ttc cag ggc cga gtc aca atg ctg gta gac aca tcc aag aac 288
Pro Lys Phe Gln Gly Arg Val Thr Met Leu Val Asp Thr Ser Lys Asn
65 70 75
cag ttc tcc ctg agg ctc tcg agt gtg aca gcc gcg gac aca gcc gta 336
Gln Phe Ser Leu Arg Leu Ser Ser Val Thr Ala Ala Asp Thr Ala Val
80 85 90
tat tac tgt gca aga gac tcg ggc tat gcc atg gac tac tgg ggc caa 384
Tyr Tyr Cys Ala Arg Asp Ser Gly Tyr Ala Met Asp Tyr Trp Gly Gln
95 100 105
ggc acc ctg gtc acc gtc tcc tca gct agc 414
Gly Thr Leu Val Thr Val Ser Ser Ala Ser
110 115

<210> 31
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<400> 31
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Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Phe Asn Ile Lys Asp Tyr
20 25 30

Tyr Met His Trp Val Lys Gln Arg Pro Gly Gln Gly Leu Glu Trp Ile
35 40 45
Gly Gly Asn Asp Pro Ala Asn Gly His Ser Met Tyr Asp Pro Lys Phe
50 55 60
Gln Gly Arg Val Thr Met Leu Val Asp Thr Ser Lys Asn Gln Phe Ser
65 70 75 80
Leu Arg Leu Ser Ser Val Thr Ala Ala Asp Thr Ala Val Tyr Tyr Cys
85 90 95
Ala Arg Asp Ser Gly Tyr Ala Met Asp Tyr Trp Gly Gln Gly Thr Leu
100 105 110
Val Thr Val Ser Ser Ala Ser
115

<210> 32
<211> 100
<212> DNA
<213> Artificial Sequence
<220>
<223> FR Shuffling primer F3EPS
<400> 32
ttcttggcca tagtatgtat gacccgaaat tccagggcag agtcacgatt actgcggacg 60
aatccacgag cacagcctac atggagctct cgagtctgag 100

<210> 33
<211> 75
<212> DNA
<213> Artificial Sequence
<220>
<223> FR Shuffling primer F3EPA
<400> 33
agaaccatgg catagcccga gtctctcgca cagaaatata cggccgagtc ctcagatctc 60
agactcgaga gctcc 75

<210> 34
<211> 20
<212> DNA
<213> Artificial Sequence
<220>
<223> Primer F3PrS

<400> 34
ttcttggcca tagtatgtat 20
<210> 35
<211> 18
<212> DNA
<213> Artificial Sequence
<220>
<223> Primer F3PrA
<400> 35
agaaccatgg catagccc 18
<210> 36
<211> 100
<212> DNA
<213> Artificial Sequence
<220>
<223> FR Shuffling primer F3VHS
<400> 36
ttcttggcca tagtatgtat gacccgaaat tccagggcag agtctcgatt accgcggacg 60
agtcaacgaa gatagcctac atggagctca acagtctgag 100
<210> 37
<211> 75
<212> DNA
<213> Artificial Sequence
<220>
<223> FR Shuffling primer F3VHA
<400> 37
agaaccatgg catagcccga gtctctcgca cagaaataaa cggccgtgtc ctcagatctc 60
agactgttga gctcc 75
<210> 38
<211> 414
<212> DNA
<213> Artificial Sequence
<220>
<221> sig-peptide
<222> (1)...(57)

<220>
<221> mat-peptide
<222> (58)...(414)
<223> Nucleotide sequence coding for version "d" of humanized H chain V region
<400> 38

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atg aaa tgc agc tgg gtc atc ttc ttc ctg atg gca gtg gtt aca ggg    48
Met Lys Cys Ser Trp Val Ile Phe Phe Leu Met Ala Val Val Thr Gly
                -15                 -10                 -5

gtt aac tca cag gtg cag ctg ttg gag tct gga gct gtg ctg gca agg    96
Val Asn Ser Gln Val Gln Leu Leu Glu Ser Gly Ala Val Leu Ala Arg
             1               5                  10

cct ggg act tcc gtg aag atc tcc tgc aag gct tcc gga ttc aac att   144
Pro Gly Thr Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Phe Asn Ile
     15                  20                  25

aaa gac tac tat atg cat tgg gta aaa cag agg cct gga cag ggt cta   192
Lys Asp Tyr Tyr Met His Trp Val Lys Gln Arg Pro Gly Gln Gly Leu
 30                  35                  40                  45

gaa tgg att ggt ggg aat gat cct gcg aat ggc cat agt atg tat gac   240
Glu Trp Ile Gly Gly Asn Asp Pro Ala Asn Gly His Ser Met Tyr Asp
                50                  55                  60

ccg aaa ttc cag ggc aga gtc acg att act gcg gac gaa tcc acg agc   288
Pro Lys Phe Gln Gly Arg Val Thr Ile Thr Ala Asp Glu Ser Thr Ser
             65                  70                  75

aca gcc tac atg gag ctc tcg agt ctg aga tct gag gac tcg gcc gta   336
Thr Ala Tyr Met Glu Leu Ser Ser Leu Arg Ser Glu Asp Ser Ala Val
         80                  85                  90

tat ttc tgt gcg aga gac tcg ggc tat gcc atg gac tac tgg ggc caa   384
Tyr Phe Cys Ala Arg Asp Ser Gly Tyr Ala Met Asp Tyr Trp Gly Gln
     95                  100                 105

ggc acc ctg gtc acc gtc tcc tca gct agc                          414
Gly Thr Leu Val Thr Val Ser Ser Ala Ser
110                 115
```

<210> 39
<211> 119
<212> PRT
<213> Artificial Sequence

<220>
<223> Amino acid sequence of version "d" of humanized H chain
<400> 39

```
Gln Val Gln Leu Leu Glu Ser Gly Ala Val Leu Ala Arg Pro Gly Thr
  1               5                  10                  15
Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Phe Asn Ile Lys Asp Tyr
             20                  25                  30
Tyr Met His Trp Val Lys Gln Arg Pro Gly Gln Gly Leu Glu Trp Ile
         35                  40                  45
Gly Gly Asn Asp Pro Ala Asn Gly His Ser Met Tyr Asp Pro Lys Phe
     50                  55                  60
Gln Gly Arg Val Thr Ile Thr Ala Asp Glu Ser Thr Ser Thr Ala Tyr
 65                  70                  75                  80
Met Glu Leu Ser Ser Leu Arg Ser Glu Asp Ser Ala Val Tyr Phe Cys
                 85                  90                  95
Ala Arg Asp Ser Gly Tyr Ala Met Asp Tyr Trp Gly Gln Gly Thr Leu
            100                 105                 110
Val Thr Val Ser Ser Ala Ser
        115
```

<210> 40
<211> 414
<212> DNA
<213> Artificial Sequence
<220>
<221> sig-peptide
<222> (1)...(57)
<220>
<221> mat-peptide
<222> (58)...(414)
<223> Nucleotide sequence coding for version "e" of humanized H chain V region
<400> 40

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atg aaa tgc agc tgg gtc atc ttc ttc ctg atg gca gtg gtt aca ggg    48
Met Lys Cys Ser Trp Val Ile Phe Phe Leu Met Ala Val Val Thr Gly
               -15                 -10                  -5
```

gtt aac tca cag gtg cag ctg ttg gag tct gga gct gtg ctg gca agg 96
Val Asn Ser Gln Val Gln Leu Leu Glu Ser Gly Ala Val Leu Ala Arg
1 5 10
cct ggg act tcc gtg aag atc tcc tgc aag gct tcc gga ttc aac att 144
Pro Gly Thr Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Phe Asn Ile
15 20 25
aaa gac tac tat atg cat tgg gta aaa cag agg cct gga cag ggt cta 192
Lys Asp Tyr Tyr Met His Trp Val Lys Gln Arg Pro Gly Gln Gly Leu
30 35 40 45
gaa tgg att ggt ggg aat gat cct gcg aat ggc cat agt atg tat gac 240
Glu Trp Ile Gly Gly Asn Asp Pro Ala Asn Gly His Ser Met Tyr Asp
50 55 60
ccg aaa ttc cag ggc aga gtc tcg att acc gcg gac gag tca acg aag 288
Pro Lys Phe Gln Gly Arg Val Ser Ile Thr Ala Asp Glu Ser Thr Lys
65 70 75
ata gcc tac atg gag ctc aac agt ctg aga tct gag gac acg gcc gtt 336
Ile Ala Tyr Met Glu Leu Asn Ser Leu Arg Ser Glu Asp Thr Ala Val
80 85 90
tat ttc tgt gcg aga gac tcg ggc tat gcc atg gac tac tgg ggc caa 384
Tyr Phe Cys Ala Arg Asp Ser Gly Tyr Ala Met Asp Tyr Trp Gly Gln
95 100 105
ggc acc ctg gtc acc gtc tcc tca gct agc 414
Gly Thr Leu Val Thr Val Ser Ser Ala Ser
110 115

<210> 41
<211> 119
<212> PRT
<213> Artificial Sequence
<220>
<223> Amino acid sequence of version "e" of humanized H chain V region
<400> 41

Gln Val Gln Leu Leu Glu Ser Gly Ala Val Leu Ala Arg Pro Gly Thr
1 5 10 15
Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Phe Asn Ile Lys Asp Tyr
20 25 30

Tyr Met His Trp Val Lys Gln Arg Pro Gly Gln Gly Leu Glu Trp Ile
35 40 45
Gly Gly Asn Asp Pro Ala Asn Gly His Ser Met Tyr Asp Pro Lys Phe
50 55 60
Gln Gly Arg Val Ser Ile Thr Ala Asp Glu Ser Thr Lys Ile Ala Tyr
65 70 75 80
Met Glu Leu Asn Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr Phe Cys
85 90 95
Ala Arg Asp Ser Gly Tyr Ala Met Asp Tyr Trp Gly Gln Gly Thr Leu
100 105 110
Val Thr Val Ser Ser Ala Ser
115

<210> 42
<211> 100
<212> DNA
<213> Artificial Sequence
<220>
<223> FR Shuffling primer F3SSS
<400> 42
ttcttggcca tagtatgtat gacccgaaat tccagggcag agtcacgatt accgcggaca 60
catccacgag cacagcctac atggagctca ggagcctgag 100

<210> 43
<211> 75
<212> DNA
<213> Artificial Sequence
<220>
<223> FR Shuffling primer F3SSA
<400> 43
agaaccatgg catagcccga gtctctcgca cagtaataca cggccgtgtc gtcagatctc 60
aggctcctga gctcc 75

<210> 44
<211> 100
<212> DNA
<213> Artificial Sequence
<220>
<223> FR Shuffling primer F3CDS

<400> 44

ttcttggcca tagtatgtat gacccgaaat tccagggcaa agccactctg actgcagacg 60

aatcctccag cacagcctac atgcaactct cgagcctacg 100

<210> 45

<211> 75

<212> DNA

<213> Artificial Sequence

<220>

<223> FR Shuffling primer F3CDA

<400> 45

agaaccatgg catagcccga gtctcttgca caagaataga ccgcagagtc ctcagatcgt 60

aggctcgaga gttgc 75

<210> 46

<211> 414

<212> DNA

<213> Artificial Sequence

<220>

<221> sig-peptide

<222> (1)...(57)

<220>

<221> mat-peptide

<222> (58)...(414)

<223> Nucleotide sequence coding for version "f" of humanized H chain V region

<400> 46

atg aaa tgc agc tgg gtc atc ttc ttc ctg atg gca gtg gtt aca ggg 48

Met Lys Cys Ser Trp Val Ile Phe Phe Leu Met Ala Val Val Thr Gly

-15 -10 -5

gtt aac tca cag gtg cag ctg ttg gag tct gga gct gtg ctg gca agg 96

Val Asn Ser Gln Val Gln Leu Leu Glu Ser Gly Ala Val Leu Ala Arg

1 5 10

cct ggg act tcc gtg aag atc tcc tgc aag gct tcc gga ttc aac att 144

Pro Gly Thr Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Phe Asn Ile

15 20 25

```
aaa gac tac tat atg cat tgg gta aaa cag agg cct gga cag ggt cta   192
Lys Asp Tyr Tyr Met His Trp Val Lys Gln Arg Pro Gly Gln Gly Leu
 30                  35                  40                  45
gaa tgg att ggt ggg aat gat cct gcg aat ggc cat agt atg tat gac   240
Glu Trp Ile Gly Gly Asn Asp Pro Ala Asn Gly His Ser Met Tyr Asp
                 50                  55                  60
ccg aaa ttc cag ggc aga gtc acg att acc gcg gac aca tcc acg agc   288
Pro Lys Phe Gln Gly Arg Val Thr Ile Thr Ala Asp Thr Ser Thr Ser
             65                  70                  75
aca gcc tac atg gag ctc agg agc ctg aga tct gac gac acg gcc gtg   336
Thr Ala Tyr Met Glu Leu Arg Ser Leu Arg Ser Asp Asp Thr Ala Val
         80                  85                  90
tat tac tgt gcg aga gac tcg ggc tat gcc atg gac tac tgg ggc caa   384
Tyr Tyr Cys Ala Arg Asp Ser Gly Tyr Ala Met Asp Tyr Trp Gly Gln
     95                  100                 105
ggc acc ctg gtc acc gtc tcc tca gct agc                          414
Gly Thr Leu Val Thr Val Ser Ser Ala Ser
110                 115
```

<210> 47
<211> 119
<212> PRT
<213> Artificial Sequence
<220>
<223> Amino acid sequence of version "f" of humanized H chain V region
<400> 47

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Gln Val Gln Leu Leu Glu Ser Gly Ala Val Leu Ala Arg Pro Gly Thr
  1               5                  10                  15
Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Phe Asn Ile Lys Asp Tyr
             20                  25                  30
Tyr Met His Trp Val Lys Gln Arg Pro Gly Gln Gly Leu Glu Trp Ile
         35                  40                  45
Gly Gly Asn Asp Pro Ala Asn Gly His Ser Met Tyr Asp Pro Lys Phe
     50                  55                  60
Gln Gly Arg Val Thr Ile Thr Ala Asp Thr Ser Thr Ser Thr Ala Tyr
 65                  70                  75                  80
```

Met Glu Leu Arg Ser Leu Arg Ser Asp Asp Thr Ala Val Tyr Tyr Cys
85 90 95
Ala Arg Asp Ser Gly Tyr Ala Met Asp Tyr Trp Gly Gln Gly Thr Leu
100 105 110
Val Thr Val Ser Ser Ala Ser
115

<210> 48
<211> 414
<212> DNA
<213> Artificial Sequence
<220>
<221> sig-peptide
<222> (1)...(57)
<220>
<221> mat-peptide
<222> (58)...(414)
<223> Nucleotide sequence coding for version "g" of humanized H chain V region

<400> 48

atg aaa tgc agc tgg gtc atc ttc ttc ctg atg gca gtg gtt aca ggg 48
Met Lys Cys Ser Trp Val Ile Phe Phe Leu Met Ala Val Val Thr Gly
-15 -10 -5
gtt aac tca cag gtg cag ctg ttg gag tct gga gct gtg ctg gca agg 96
Val Asn Ser Gln Val Gln Leu Leu Glu Ser Gly Ala Val Leu Ala Arg
1 5 10
cct ggg act tcc gtg aag atc tcc tgc aag gct tcc gga ttc aac att 144
Pro Gly Thr Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Phe Asn Ile
15 20 25
aaa gac tac tat atg cat tgg gta aaa cag agg cct gga cag ggt cta 192
Lys Asp Tyr Tyr Met His Trp Val Lys Gln Arg Pro Gly Gln Gly Leu
30 35 40 45
gaa tgg att ggt ggg aat gat cct gcg aat ggc cat agt atg tat gac 240
Glu Trp Ile Gly Gly Asn Asp Pro Ala Asn Gly His Ser Met Tyr Asp
50 55 60

ccg aaa ttc cag ggc aaa gcc act ctg act gca gac gaa tcc tcc agc 288
Pro Lys Phe Gln Gly Lys Ala Thr Leu Thr Ala Asp Glu Ser Ser Ser
65 70 75
aca gcc tac atg caa ctc tcg agc cta cga tct gag gac tct gcg gtc 336
Thr Ala Tyr Met Gln Leu Ser Ser Leu Arg Ser Glu Asp Ser Ala Val
80 85 90
tat tct tgt gca aga gac tcg ggc tat gcc atg gac tac tgg ggc caa 384
Tyr Ser Cys Ala Arg Asp Ser Gly Tyr Ala Met Asp Tyr Trp Gly Gln
95 100 105
ggc acc ctg gtc acc gtc tcc tca gct agc 414
Gly Thr Leu Val Thr Val Ser Ser Ala Ser
110 115

<210> 49
<211> 119
<212> PRT
<213> Artificial Sequence
<220>
<223> Amino acid sequence of version "g" of humanized H chain V region
<400> 49

Gln Val Gln Leu Leu Glu Ser Gly Ala Val Leu Ala Arg Pro Gly Thr
1 5 10 15
Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Phe Asn Ile Lys Asp Tyr
20 25 30
Tyr Met His Trp Val Lys Gln Arg Pro Gly Gln Gly Leu Glu Trp Ile
35 40 45
Gly Gly Asn Asp Pro Ala Asn Gly His Ser Met Tyr Asp Pro Lys Phe
50 55 60
Gln Gly Lys Ala Thr Leu Thr Ala Asp Glu Ser Ser Ser Thr Ala Tyr
65 70 75 80
Met Gln Leu Ser Ser Leu Arg Ser Glu Asp Ser Ala Val Tyr Ser Cys
85 90 95
Ala Arg Asp Ser Gly Tyr Ala Met Asp Tyr Trp Gly Gln Gly Thr Leu
100 105 110
Val Thr Val Ser Ser Ala Ser
115

<210> 50

<211> 100
<212> DNA
<213> Artificial Sequence
<220>
<223> FR Shuffling primer F3ADS
<400> 50
ttcttggcca tagtatgtat gacccgaaat tccagggccg cgtcaccatg tcagccgaca 60
agtcctccag cgccgcctat ttacagtgga ccagccttaa 100
<210> 51
<211> 75
<212> DNA
<213> Artificial Sequence
<220>
<223> FR Shuffling primer F3ADA
<400> 51
agaaccatgg catagcccga gtctctcgcg cagaaatata tggcggtgtc cgaggcctta 60
aggctggtcc actgt 75
<210> 52
<211> 414
<212> DNA
<213> Artificial Sequence
<220>
<221> sig-peptide
<222> (1)...(57)
<220>
<221> mat-peptide
<222> (58)...(414)
<223> Nucleotide sequence coding for version "h" of humanized H chain
<400> 52
atg aaa tgc agc tgg gtc atc ttc ttc ctg atg gca gtg gtt aca ggg 48
Met Lys Cys Ser Trp Val Ile Phe Phe Leu Met Ala Val Val Thr Gly
-15 -10 -5
gtt aac tca cag gtg cag ctg ttg gag tct gga gct gtg ctg gca agg 96
Val Asn Ser Gln Val Gln Leu Leu Glu Ser Gly Ala Val Leu Ala Arg
1 5 10

cct ggg act tcc gtg aag atc tcc tgc aag gct tcc gga ttc aac att 144
Pro Gly Thr Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Phe Asn Ile
15 20 25
aaa gac tac tat atg cat tgg gta aaa cag agg cct gga cag ggt cta 192
Lys Asp Tyr Tyr Met His Trp Val Lys Gln Arg Pro Gly Gln Gly Leu
30 35 40 45
gaa tgg att ggt ggg aat gat cct gcg aat ggc cat agt atg tat gac 240
Glu Trp Ile Gly Gly Asn Asp Pro Ala Asn Gly His Ser Met Tyr Asp
50 55 60
ccg aaa ttc cag ggc cgc gtc acc atg tca gcc gac aag tcc tcc agc 288
Pro Lys Phe Gln Gly Arg Val Thr Met Ser Ala Asp Lys Ser Ser Ser
65 70 75
gcc gcc tat tta cag tgg acc agc ctt aag gcc tcg gac acc gcc ata 336
Ala Ala Tyr Leu Gln Trp Thr Ser Leu Lys Ala Ser Asp Thr Ala Ile
80 85 90
tat ttc tgc gcg aga gac tcg ggc tat gcc atg gac tac tgg ggc caa 384
Tyr Phe Cys Ala Arg Asp Ser Gly Tyr Ala Met Asp Tyr Trp Gly Gln
95 100 105
ggc acc ctg gtc acc gtc tcc tca gct agc 414
Gly Thr Leu Val Thr Val Ser Ser Ala Ser
110 115

<210> 53
<211> 119
<212> PRT
<213> Artificial Sequence
<220>
<223> Amino acid sequence of version "h" of humanized H chain V region
<400> 53

Gln Val Gln Leu Leu Glu Ser Gly Ala Val Leu Ala Arg Pro Gly Thr
1 5 10 15
Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Phe Asn Ile Lys Asp Tyr
20 25 30
Tyr Met His Trp Val Lys Gln Arg Pro Gly Gln Gly Leu Glu Trp Ile
35 40 45
Gly Gly Asn Asp Pro Ala Asn Gly His Ser Met Tyr Asp Pro Lys Phe
50 55 60

Gln Gly Arg Val Thr Met Ser Ala Asp Lys Ser Ser Ser Ala Ala Tyr
65 70 75 80
Leu Gln Trp Thr Ser Leu Lys Ala Ser Asp Thr Ala Ile Tyr Phe Cys
85 90 95
Ala Arg Asp Ser Gly Tyr Ala Met Asp Tyr Trp Gly Gln Gly Thr Leu
100 105 110
Val Thr Val Ser Ser Ala Ser
115

<210> 54
<211> 100
<212> DNA
<213> Artificial Sequence
<220>
<223> FR Shuffling primer F3MMS
<400> 54
ttcttggcca tagtatgtat gacccgaaat tccagggcag agtcacgatt accgcggaca 60
catcgacgag cacagtcttc atggaactga gcagcctgag 100

<210> 55
<211> 75
<212> DNA
<213> Artificial Sequence
<220>
<223> FR Shuffling primer F3MMA
<400> 55
agaaccatgg catagcccga gtctctcgca cagtaataca cggccgtgtc ttcagatctc 60
aggctgctca gttcc 75

<210> 56
<211> 100
<212> DNA
<213> Artificial Sequence
<220>
<223> FR Shuffling primer F3BMS
<400> 56
ttcttggcca tagtatgtat gacccgaaat tccagggcag agtcaccttt accgcggaca 60
catccgcgaa cacagcctac atggagttga ggagcctcag 100

<210> 57

<211> 75
<212> DNA
<213> Artificial Sequence
<220>
<223> FR Shuffling primer F3BMA
<400> 57
agaaccatgg catagcccga gtctctcgca caataataaa cagccgtgtc tgcagatctg 60
aggctcctca actcc 75
<210> 58
<211> 414
<212> DNA
<213> Artificial Sequence
<220>
<221> sig-peptide
<222> (1)...(57)
<220>
<221> mat-peptide
<222> (58)...(414)
<223> Nucleotide sequence coding for version "i" of humanized H chain V region
<400> 58

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atg aaa tgc agc tgg gtc atc ttc ttc ctg atg gca gtg gtt aca ggg    48
Met Lys Cys Ser Trp Val Ile Phe Phe Leu Met Ala Val Val Thr Gly
                -15                 -10                 -5

gtt aac tca cag gtg cag ctg ttg gag tct gga gct gtg ctg gca agg    96
Val Asn Ser Gln Val Gln Leu Leu Glu Ser Gly Ala Val Leu Ala Arg
            1               5                  10

cct ggg act tcc gtg aag atc tcc tgc aag gct tcc gga ttc aac att   144
Pro Gly Thr Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Phe Asn Ile
     15                  20                  25

aaa gac tac tat atg cat tgg gta aaa cag agg cct gga cag ggt cta   192
Lys Asp Tyr Tyr Met His Trp Val Lys Gln Arg Pro Gly Gln Gly Leu
 30                  35                  40                  45

gaa tgg att ggt ggg aat gat cct gcg aat ggc cat agt atg tat gac   240
Glu Trp Ile Gly Gly Asn Asp Pro Ala Asn Gly His Ser Met Tyr Asp
                50                  55                  60
```

ccg aaa ttc cag ggc aga gtc acg att acc gcg gac aca tcg acg agc 288
Pro Lys Phe Gln Gly Arg Val Thr Ile Thr Ala Asp Thr Ser Thr Ser
65 70 75

aca gtc ttc atg gaa ctg agc agc ctg aga tct gaa gac acg gcc gtg 336
Thr Val Phe Met Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Val
80 85 90

tat tac tgt gcg aga gac tcg ggc tat gcc atg gac tac tgg ggc caa 384
Tyr Tyr Cys Ala Arg Asp Ser Gly Tyr Ala Met Asp Tyr Trp Gly Gln
95 100 105

ggc acc ctg gtc acc gtc tcc tca gct agc 414
Gly Thr Leu Val Thr Val Ser Ser Ala Ser
110 115

<210> 59
<211> 119
<212> PRT
<213> Artificial Sequence
<220>
<223> Amino acid sequence of version "i" of humanized H chain V region
<400> 59

Gln Val Gln Leu Leu Glu Ser Gly Ala Val Leu Ala Arg Pro Gly Thr
1 5 10 15

Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Phe Asn Ile Lys Asp Tyr
20 25 30

Tyr Met His Trp Val Lys Gln Arg Pro Gly Gln Gly Leu Glu Trp Ile
35 40 45

Gly Gly Asn Asp Pro Ala Asn Gly His Ser Met Tyr Asp Pro Lys Phe
50 55 60

Gln Gly Arg Val Thr Ile Thr Ala Asp Thr Ser Thr Ser Thr Val Phe
65 70 75 80

Met Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr Tyr Cys
85 90 95

Ala Arg Asp Ser Gly Tyr Ala Met Asp Tyr Trp Gly Gln Gly Thr Leu
100 105 110

Val Thr Val Ser Ser Ala Ser
115

<210> 60

<211> 414
<212> DNA
<213> Artificial Sequence
<220>
<221> sig-peptide
<222> (1)...(57)
<220>
<221> mat-peptide
<222> (58)...(414)
<223> Nucleotide sequence coding for version "j" of humanized H chain V region
<400> 60

```
atg aaa tgc agc tgg gtc atc ttc ttc ctg atg gca gtg gtt aca ggg    48
Met Lys Cys Ser Trp Val Ile Phe Phe Leu Met Ala Val Val Thr Gly
                -15                 -10                 -5

gtt aac tca cag gtg cag ctg ttg gag tct gga gct gtg ctg gca agg    96
Val Asn Ser Gln Val Gln Leu Leu Glu Ser Gly Ala Val Leu Ala Arg
            1               5                  10

cct ggg act tcc gtg aag atc tcc tgc aag gct tcc gga ttc aac att   144
Pro Gly Thr Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Phe Asn Ile
     15                  20                  25

aaa gac tac tat atg cat tgg gta aaa cag agg cct gga cag ggt cta   192
Lys Asp Tyr Tyr Met His Trp Val Lys Gln Arg Pro Gly Gln Gly Leu
 30                  35                  40                  45

gaa tgg att ggt ggg aat gat cct gcg aat ggc cat agt atg tat gac   240
Glu Trp Ile Gly Gly Asn Asp Pro Ala Asn Gly His Ser Met Tyr Asp
                50                  55                  60

ccg aaa ttc cag ggc aga gtc acc ttt acc gcg gac aca tcc gcg aac   288
Pro Lys Phe Gln Gly Arg Val Thr Phe Thr Ala Asp Thr Ser Ala Asn
            65                  70                  75

aca gcc tac atg gag ttg agg agc ctc aga tct gca gac acg gct gtt   336
Thr Ala Tyr Met Glu Leu Arg Ser Leu Arg Ser Ala Asp Thr Ala Val
        80                  85                  90

tat tat tgt gcg aga gac tcg ggc tat gcc atg gac tac tgg ggc caa   384
Tyr Tyr Cys Ala Arg Asp Ser Gly Tyr Ala Met Asp Tyr Trp Gly Gln
    95                  100                 105
```

ggc acc ctg gtc acc gtc tcc tca gct agc 414
Gly Thr Leu Val Thr Val Ser Ser Ala Ser
110 115

<210> 61
<211> 119
<212> PRT
<213> Artificial Sequence
<220>
<223> Amino acid sequence of version "j" of humanized H chain V region
<400> 61

Gln Val Gln Leu Leu Glu Ser Gly Ala Val Leu Ala Arg Pro Gly Thr
1 5 10 15
Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Phe Asn Ile Lys Asp Tyr
20 25 30
Tyr Met His Trp Val Lys Gln Arg Pro Gly Gln Gly Leu Glu Trp Ile
35 40 45
Gly Gly Asn Asp Pro Ala Asn Gly His Ser Met Tyr Asp Pro Lys Phe
50 55 60
Gln Gly Arg Val Thr Phe Thr Ala Asp Thr Ser Ala Asn Thr Ala Tyr
65 70 75 80
Met Glu Leu Arg Ser Leu Arg Ser Ala Asp Thr Ala Val Tyr Tyr Cys
85 90 95
Ala Arg Asp Ser Gly Tyr Ala Met Asp Tyr Trp Gly Gln Gly Thr Leu
100 105 110
Val Thr Val Ser Ser Ala Ser
115

<210> 62
<211> 79
<212> DNA
<213> Artificial Sequence
<220>
<223> FR shuffling primer F2MPS
<400> 62

ttctatgcat tgggtgcgcc aggctccagg acagggcctg gagtggatgg gagggaatga 60
tcctgcgaat ggccattct 79

<210> 63
<211> 79
<212> DNA
<213> Artificial Sequence
<220>
<223> FR shuffling primer F2MPA
<400> 63
agaatggcca ttcgcaggat cattccctcc catccactcc aggccctgtc ctggagcctg 60
gcgcacccaa tgcatagaa 79
<210> 64
<211> 414
<212> DNA
<213> Artificial Sequence
<220>
<221> sig-peptide
<222> (1)...(57)
<220>
<221> mat-peptide
<222> (58)...(414)
<223> Nucleotide sequence coding for version "b1" of humanized H chain V region
<400> 64

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atg aaa tgc agc tgg gtc atc ttc ttc ctg atg gca gtg gtt aca ggg    48
Met Lys Cys Ser Trp Val Ile Phe Phe Leu Met Ala Val Val Thr Gly
               -15                 -10                  -5

gtt aac tca cag gtg cag ctg ttg gag tct gga gct gtg ctg gca agg    96
Val Asn Ser Gln Val Gln Leu Leu Glu Ser Gly Ala Val Leu Ala Arg
            1               5                  10

cct ggg act tcc gtg aag atc tcc tgc aag gct tcc gga ttc aac att   144
Pro Gly Thr Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Phe Asn Ile
     15                  20                  25

aaa gac tac tat atg cat tgg gtg cgc cag gct cca gga cag ggc ctg   192
Lys Asp Tyr Tyr Met His Trp Val Arg Gln Ala Pro Gly Gln Gly Leu
 30                  35                  40                  45
```

gag tgg atg gga ggg aat gat cct gcg aat ggc cat agt atg tat gac 240
Glu Trp Met Gly Gly Asn Asp Pro Ala Asn Gly His Ser Met Tyr Asp
50 55 60
ccg aaa ttc cag ggc cga gtc aca atc act gca gac aca tcc acg aac 288
Pro Lys Phe Gln Gly Arg Val Thr Ile Thr Ala Asp Thr Ser Thr Asn
65 70 75
aca gcc tac atg gag ctc tcg agt ctg aga tct gag gac aca gcc att 336
Thr Ala Tyr Met Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Ile
80 85 90
tat tac tgt gca aga gac tcg ggc tat gcc atg gac tac tgg ggc caa 384
Tyr Tyr Cys Ala Arg Asp Ser Gly Tyr Ala Met Asp Tyr Trp Gly Gln
95 100 105
ggc acc ctg gtc acc gtc tcc tca gct agc 414
Gly Thr Leu Val Thr Val Ser Ser Ala Ser
110 115

<210> 65
<211> 119
<212> PRT
<213> Artificial Sequence
<220>
<223> Amino acid sequence of version "b1" of humanized H chain V region
<400> 65

Gln Val Gln Leu Leu Glu Ser Gly Ala Val Leu Ala Arg Pro Gly Thr
1 5 10 15
Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Phe Asn Ile Lys Asp Tyr
20 25 30
Tyr Met His Trp Val Arg Gln Ala Pro Gly Gln Gly Leu Glu Trp Met
35 40 45
Gly Gly Asn Asp Pro Ala Asn Gly His Ser Met Tyr Asp Pro Lys Phe
50 55 60
Gln Gly Arg Val Thr Ile Thr Ala Asp Thr Ser Thr Asn Thr Ala Tyr
65 70 75 80
Met Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Ile Tyr Tyr Cys
85 90 95
Ala Arg Asp Ser Gly Tyr Ala Met Asp Tyr Trp Gly Gln Gly Thr Leu
100 105 110

Val Thr Val Ser Ser Ala Ser
115

<210> 66
<211> 414
<212> DNA
<213> Artificial Sequence
<220>
<221> sig-peptide
<222> (1)...(57)
<220>
<221> mat-peptide
<222> (58)...(414)
<223> Nucleotide sequence coding for version "d1" of humanized H chain V region
<400> 66

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atg aaa tgc agc tgg gtc atc ttc ttc ctg atg gca gtg gtt aca ggg    48
Met Lys Cys Ser Trp Val Ile Phe Phe Leu Met Ala Val Val Thr Gly
               -15                 -10                 -5

gtt aac tca cag gtg cag ctg ttg gag tct gga gct gtg ctg gca agg    96
Val Asn Ser Gln Val Gln Leu Leu Glu Ser Gly Ala Val Leu Ala Arg
            1               5                  10

cct ggg act tcc gtg aag atc tcc tgc aag gct tcc gga ttc aac att   144
Pro Gly Thr Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Phe Asn Ile
     15                  20                  25

aaa gac tac tat atg cat tgg gtg cgc cag gct cca gga cag ggc ctg   192
Lys Asp Tyr Tyr Met His Trp Val Arg Gln Ala Pro Gly Gln Gly Leu
 30                  35                  40                  45

gag tgg atg gga ggg aat gat cct gcg aat ggc cat agt atg tat gac   240
Glu Trp Met Gly Gly Asn Asp Pro Ala Asn Gly His Ser Met Tyr Asp
                50                  55                  60

ccg aaa ttc cag ggc aga gtc acg att act gcg gac gaa tcc acg agc   288
Pro Lys Phe Gln Gly Arg Val Thr Ile Thr Ala Asp Glu Ser Thr Ser
            65                  70                  75

aca gcc tac atg gag ctc tcg agt ctg aga tct gag gac tcg gcc gta   336
Thr Ala Tyr Met Glu Leu Ser Ser Leu Arg Ser Glu Asp Ser Ala Val
         80                  85                  90
```

tat ttc tgt gcg aga gac tcg ggc tat gcc atg gac tac tgg ggc caa 384
Tyr Phe Cys Ala Arg Asp Ser Gly Tyr Ala Met Asp Tyr Trp Gly Gln
95 100 105
ggc acc ctg gtc acc gtc tcc tca gct agc 414
Gly Thr Leu Val Thr Val Ser Ser Ala Ser
110 115

<210> 67
<211> 119
<212> PRT
<213> Artificial Sequence
<220>
<223> Amino acid sequence of version "d1" of humanized H chain V region
<400> 67

Gln Val Gln Leu Leu Glu Ser Gly Ala Val Leu Ala Arg Pro Gly Thr
1 5 10 15
Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Phe Asn Ile Lys Asp Tyr
20 25 30
Tyr Met His Trp Val Arg Gln Ala Pro Gly Gln Gly Leu Glu Trp Met
35 40 45
Gly Gly Asn Asp Pro Ala Asn Gly His Ser Met Tyr Asp Pro Lys Phe
50 55 60
Gln Gly Arg Val Thr Ile Thr Ala Asp Glu Ser Thr Ser Thr Ala Tyr
65 70 75 80
Met Glu Leu Ser Ser Leu Arg Ser Glu Asp Ser Ala Val Tyr Phe Cys
85 90 95
Ala Arg Asp Ser Gly Tyr Ala Met Asp Tyr Trp Gly Gln Gly Thr Leu
100 105 110
Val Thr Val Ser Ser Ala Ser
115

<210> 68
<211> 79
<212> DNA
<213> Artificial Sequence
<220>
<223> FR shuffling primer F2VHS

<400> 68

ttctatgcat tgggtgcgac aggcccctgg acaagggctt gagtggattg gagggaatga 60

tcctgcgaat ggccatctt 79

<210> 69

<211> 79

<212> DNA

<213> Artificial Sequence

<220>

<223> FR shuffling primer F2VHA

<400> 69

aagatggcca ttcgcaggat cattccctcc aatccactca agcccttgtc caggggcctg 60

tcgcacccaa tgcatagaa 79

<210> 70

<211> 414

<212> DNA

<213> Artificial Sequence

<220>

<221> sig-peptide

<222> (1)...(57)

<220>

<221> mat-peptide

<222> (58)...(414)

<223> Nucleotide sequence coding for version "b3" of humanized H chain V region

<400> 70

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atg aaa tgc agc tgg gtc atc ttc ttc ctg atg gca gtg gtt aca ggg     48
Met Lys Cys Ser Trp Val Ile Phe Phe Leu Met Ala Val Val Thr Gly
                -15                 -10                  -5
gtt aac tca cag gtg cag ctg ttg gag tct gga gct gtg ctg gca agg     96
Val Asn Ser Gln Val Gln Leu Leu Glu Ser Gly Ala Val Leu Ala Arg
             1                   5                  10
cct ggg act tcc gtg aag atc tcc tgc aag gct tcc gga ttc aac att    144
Pro Gly Thr Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Phe Asn Ile
     15                  20                  25
```

aaa gac tac tat atg cat tgg gtg cga cag gcc cct gga caa ggg ctt 192
Lys Asp Tyr Tyr Met His Trp Val Arg Gln Ala Pro Gly Gln Gly Leu
30 35 40 45
gag tgg att gga ggg aat gat cct gcg aat ggc cat agt atg tat gac 240
Glu Trp Ile Gly Gly Asn Asp Pro Ala Asn Gly His Ser Met Tyr Asp
50 55 60
ccg aaa ttc cag ggc cga gtc aca atc act gca gac aca tcc acg aac 288
Pro Lys Phe Gln Gly Arg Val Thr Ile Thr Ala Asp Thr Ser Thr Asn
65 70 75
aca gcc tac atg gag ctc tcg agt ctg aga tct gag gac aca gcc att 336
Thr Ala Tyr Met Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Ile
80 85 90
tat tac tgt gca aga gac tcg ggc tat gcc atg gac tac tgg ggc caa 384
Tyr Tyr Cys Ala Arg Asp Ser Gly Tyr Ala Met Asp Tyr Trp Gly Gln
95 100 105
ggc acc ctg gtc acc gtc tcc tca gct agc 414
Gly Thr Leu Val Thr Val Ser Ser Ala Ser
110 115

<210> 71
<211> 119
<212> PRT
<213> Artificial Sequence
<220>
<223> Amino acid sequence of version "b3" of humanized H chain V region
<400> 71

Gln Val Gln Leu Leu Glu Ser Gly Ala Val Leu Ala Arg Pro Gly Thr
1 5 10 15
Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Phe Asn Ile Lys Asp Tyr
20 25 30
Tyr Met His Trp Val Arg Gln Ala Pro Gly Gln Gly Leu Glu Trp Ile
35 40 45
Gly Gly Asn Asp Pro Ala Asn Gly His Ser Met Tyr Asp Pro Lys Phe
50 55 60
Gln Gly Arg Val Thr Ile Thr Ala Asp Thr Ser Thr Asn Thr Ala Tyr
65 70 75 80

Met Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Ile Tyr Tyr Cys
85 90 95
Ala Arg Asp Ser Gly Tyr Ala Met Asp Tyr Trp Gly Gln Gly Thr Leu
100 105 110
Val Thr Val Ser Ser Ala Ser
115

<210> 72
<211> 414
<212> DNA
<213> Artificial Sequence
<220>
<221> sig-peptide
<222> (1)...(57)
<220>
<221> mat-peptide
<222> (58)...(414)
<223> Nucleotide sequence coding for version "d3" of humanized H chain V region

<400> 72

atg aaa tgc agc tgg gtc atc ttc ttc ctg atg gca gtg gtt aca ggg 48
Met Lys Cys Ser Trp Val Ile Phe Phe Leu Met Ala Val Val Thr Gly
-15 -10 -5
gtt aac tca cag gtg cag ctg ttg gag tct gga gct gtg ctg gca agg 96
Val Asn Ser Gln Val Gln Leu Leu Glu Ser Gly Ala Val Leu Ala Arg
1 5 10
cct ggg act tcc gtg aag atc tcc tgc aag gct tcc gga ttc aac att 144
Pro Gly Thr Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Phe Asn Ile
15 20 25
aaa gac tac tat atg cat tgg gtg cga cag gcc cct gga caa ggg ctt 192
Lys Asp Tyr Tyr Met His Trp Val Arg Gln Ala Pro Gly Gln Gly Leu
30 35 40 45
gag tgg att gga ggg aat gat cct gcg aat ggc cat agt atg tat gac 240
Glu Trp Ile Gly Gly Asn Asp Pro Ala Asn Gly His Ser Met Tyr Asp
50 55 60

ccg aaa ttc cag ggc aga gtc acg att act gcg gac gaa tcc acg agc 288
Pro Lys Phe Gln Gly Arg Val Thr Ile Thr Ala Asp Glu Ser Thr Ser
65 70 75
aca gcc tac atg gag ctc tcg agt ctg aga tct gag gac tcg gcc gta 336
Thr Ala Tyr Met Glu Leu Ser Ser Leu Arg Ser Glu Asp Ser Ala Val
80 85 90
tat ttc tgt gcg aga gac tcg ggc tat gcc atg gac tac tgg ggc caa 384
Tyr Phe Cys Ala Arg Asp Ser Gly Tyr Ala Met Asp Tyr Trp Gly Gln
95 100 105
ggc acc ctg gtc acc gtc tcc tca gct agc 414
Gly Thr Leu Val Thr Val Ser Ser Ala Ser
110 115

<210> 73
<211> 119
<212> PRT
<213> Artificial Sequence
<220>
<223> Amino acid sequence of version "d3" of humanized H chain V region
<400> 73

Gln Val Gln Leu Leu Glu Ser Gly Ala Val Leu Ala Arg Pro Gly Thr
1 5 10 15
Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Phe Asn Ile Lys Asp Tyr
20 25 30
Tyr Met His Trp Val Arg Gln Ala Pro Gly Gln Gly Leu Glu Trp Ile
35 40 45
Gly Gly Asn Asp Pro Ala Asn Gly His Ser Met Tyr Asp Pro Lys Phe
50 55 60
Gln Gly Arg Val Thr Ile Thr Ala Asp Glu Ser Thr Ser Thr Ala Tyr
65 70 75 80
Met Glu Leu Ser Ser Leu Arg Ser Glu Asp Ser Ala Val Tyr Phe Cys
85 90 95
Ala Arg Asp Ser Gly Tyr Ala Met Asp Tyr Trp Gly Gln Gly Thr Leu
100 105 110
Val Thr Val Ser Ser Ala Ser
115

<210> 74

<211> 98
<212> DNA
<213> Artificial Sequence
<220>
<223> FR shuffling vector h5Lv1S
<400> 74
gtctagatct ccaccatgag ggcccctgct cagtttttg ggatcttgtt gctctggttt 60
ccagggatcc gatgtgacat ccagatgacc cagtctcc 98
<210> 75
<211> 98
<212> DNA
<213> Artificial Sequence
<220>
<223> FR shuffling vector h5Lv4S
<400> 75
ttggcagatg gggtcccatc aaggttcagt ggctccggat ctggtaccga tttcactctc 60
accatctcga gtctgcaacc tgaagatttt gcaactta 98
<210> 76
<211> 98
<212> DNA
<213> Artificial Sequence
<220>
<223> FR shuffling vector h5Lv2A
<400> 76
cttaagaagc ttttaatgtc ctgtgaggcc ttgcacgtga tggtgactct gtctcctaca 60
gatgcagaca gggaggatgg agactgggtc atctggat 98
<210> 77
<211> 98
<212> DNA
<213> Artificial Sequence
<220>
<223> FR shuffling vector h5Lv3A
<400> 77
gatgggaccc catctgccaa actagttgca taatagatca ggagcttagg ggctttccct 60
ggtttctgct gataccaact taagaagctt ttaatgtc 98

<210> 78
<211> 94
<212> DNA
<213> Artificial Sequence
<220>
<223> FR shuffling vector h5Lv5A
<400> 78
tgttcgtacg tttgatctcc accttggtcc ctccgccgaa cgtgtacggg ctctcaccat 60
gctgcagaca gtagtaagtt gcaaaatctt cagg 94
<210> 79
<211> 20
<212> DNA
<213> Artificial Sequence
<220>
<223> Primer h5LvS
<400> 79
gtctagatct ccaccatgag 20
<210> 80
<211> 19
<212> DNA
<213> Artificial Sequence
<220>
<223> Primer h5LvA
<400> 80
tgttcgtacg tttgatctc 19
<210> 81
<211> 381
<212> DNA
<213> Artificial Sequence
<220>
<221> sig-peptide
<222> (1)...(60)
<220>
<221> mat-peptide
<222> (61)...(381)

<223> Nucleotide sequence coding for version "a" of humanized L chain V region

<400> 81

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atg agg gcc cct gct cag ttt ttt ggg atc ttg ttg ctc tgg ttt cca    48
Met Arg Ala Pro Ala Gln Phe Phe Gly Ile Leu Leu Leu Trp Phe Pro
-20                 -15                 -10                 -5
ggg atc cga tgt gac atc cag atg acc cag tct cca tcc tcc ctg tct    96
Gly Ile Arg Cys Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser
                 1               5                  10
gca tct gta gga gac aga gtc acc atc acg tgc aag gcc tca cag gac   144
Ala Ser Val Gly Asp Arg Val Thr Ile Thr Cys Lys Ala Ser Gln Asp
         15                  20                  25
att aaa agc ttc tta agt tgg tat cag cag aaa cca ggg aaa gcc cct   192
Ile Lys Ser Phe Leu Ser Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro
     30                  35                  40
aag ctc ctg atc tat tat gca act agt ttg gca gat ggg gtc cca tca   240
Lys Leu Leu Ile Tyr Tyr Ala Thr Ser Leu Ala Asp Gly Val Pro Ser
 45                  50                  55                  60
agg ttc agt ggc tcc gga tct ggt acc gat ttc act ctc acc atc tcg   288
Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser
                 65                  70                  75
agt ctg caa cct gaa gat ttt gca act tac tac tgt ctg cag cat ggt   336
Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Leu Gln His Gly
             80                  85                  90
gag agc ccg tac acg ttc ggc gga ggg acc aag gtg gag atc aaa       381
Glu Ser Pro Tyr Thr Phe Gly Gly Gly Thr Lys Val Glu Ile Lys
         95                  100                 105
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<210> 82

<211> 107

<212> PRT

<213> Artificial Sequence

<220>

<223> Amino acid sequence of version "a" of humanized L chain V region

<400> 82

Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
1 5 10 15
Asp Arg Val Thr Ile Thr Cys Lys Ala Ser Gln Asp Ile Lys Ser Phe
20 25 30
Leu Ser Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
35 40 45
Tyr Tyr Ala Thr Ser Leu Ala Asp Gly Val Pro Ser Arg Phe Ser Gly
50 55 60
Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro
65 70 75 80
Glu Asp Phe Ala Thr Tyr Tyr Cys Leu Gln His Gly Glu Ser Pro Tyr
85 90 95
Thr Phe Gly Gly Gly Thr Lys Val Glu Ile Lys
100 105

<210> 83
<211> 77
<212> DNA
<213> Artificial Sequence
<220>
<223> FR shuffling primer F3SS
<400> 83
gtctggtacc gattacactc tcaccatctc gagcctccag cctgaagatt ttgcaactta 60
ctattgtctg cagaaca 77
<210> 84
<211> 77
<212> DNA
<213> Artificial Sequence
<220>
<223> FR shuffling primer F3SA
<400> 84
tgttctgcag acaatagtaa gttgcaaaat cttcaggctg gaggctcgag atggtgagag 60
tgtaatcggt accagac 77
<210> 85
<211> 77
<212> DNA
<213> Artificial Sequence

<220>
<223> FR shuffling primer F3RS
<400> 85
gtctggtacc gattacactc tcaccatctc gagcctccag cctgaagata ttgcaactta 60
ctattgtctg cagaaca 77
<210> 86
<211> 77
<212> DNA
<213> Artificial Sequence
<220>
<223> FR shuffling primer F3RA
<400> 86
tgttctgcag acaatagtaa gttgcaatat cttcaggctg gaggctcgag atggtgagag 60
tgtaatcggt accagac 77
<210> 87
<211> 381
<212> DNA
<213> Artificial Sequence
<220>
<221> sig-peptide
<222> (1)...(60)
<220>
<221> mat-peptide
<222> (61)...(381)
<223> Nucleotide sequence coding for version "b" of humanized L chain V region
<400> 87
atg agg gcc cct gct cag ttt ttt ggg atc ttg ttg ctc tgg ttt cca 48
Met Arg Ala Pro Ala Gln Phe Phe Gly Ile Leu Leu Leu Trp Phe Pro
-20 -15 -10 -5
ggg atc cga tgt gac atc cag atg acc cag tct cca tcc tcc ctg tct 96
Gly Ile Arg Cys Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser
1 5 10
gca tct gta gga gac aga gtc acc atc acg tgc aag gcc tca cag gac 144
Ala Ser Val Gly Asp Arg Val Thr Ile Thr Cys Lys Ala Ser Gln Asp
15 20 25

att aaa agc ttc tta agt tgg tat cag cag aaa cca ggg aaa gcc cct 192
Ile Lys Ser Phe Leu Ser Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro
30 35 40
aag ctc ctg atc tat tat gca act agt ttg gca gat ggg gtc cca tca 240
Lys Leu Leu Ile Tyr Tyr Ala Thr Ser Leu Ala Asp Gly Val Pro Ser
45 50 55 60
agg ttc agt ggc tcc gga tct ggt acc gat tac act ctc acc atc tcg 288
Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Tyr Thr Leu Thr Ile Ser
65 70 75
agc ctc cag cct gaa gat ttt gca act tac tat tgt ctg cag cat ggt 336
Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Leu Gln His Gly
80 85 90
gag agc ccg tac acg ttc ggc gga ggg acc aag gtg gag atc aaa 381
Glu Ser Pro Tyr Thr Phe Gly Gly Gly Thr Lys Val Glu Ile Lys
95 100 105

<210> 88
<211> 107
<212> PRT
<213> Artificial Sequence
<220>
<223> Amino acid sequence of version "b" of humanized L chain V region
<400> 88

Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
1 5 10 15
Asp Arg Val Thr Ile Thr Cys Lys Ala Ser Gln Asp Ile Lys Ser Phe
20 25 30
Leu Ser Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
35 40 45
Tyr Tyr Ala Thr Ser Leu Ala Asp Gly Val Pro Ser Arg Phe Ser Gly
50 55 60
Ser Gly Ser Gly Thr Asp Tyr Thr Leu Thr Ile Ser Ser Leu Gln Pro
65 70 75 80
Glu Asp Phe Ala Thr Tyr Tyr Cys Leu Gln His Gly Glu Ser Pro Tyr
85 90 95
Thr Phe Gly Gly Gly Thr Lys Val Glu Ile Lys
100 105

<210> 89
<211> 381
<212> DNA
<213> Artificial Sequence
<220>
<221> sig-peptide
<222> (1)...(60)
<220>
<221> mat-peptide
<222> (61)...(381)
<223> Nucleotide sequence coding for version "c" of humanized L chain V region
<400> 89

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atg agg gcc cct gct cag ttt ttt ggg atc ttg ttg ctc tgg ttt cca    48
Met Arg Ala Pro Ala Gln Phe Phe Gly Ile Leu Leu Leu Trp Phe Pro
-20                 -15                 -10                 -5
ggg atc cga tgt gac atc cag atg acc cag tct cca tcc tcc ctg tct    96
Gly Ile Arg Cys Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser
                 1               5                  10
gca tct gta gga gac aga gtc acc atc acg tgc aag gcc tca cag gac   144
Ala Ser Val Gly Asp Arg Val Thr Ile Thr Cys Lys Ala Ser Gln Asp
         15                  20                  25
att aaa agc ttc tta agt tgg tat cag cag aaa cca ggg aaa gcc cct   192
Ile Lys Ser Phe Leu Ser Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro
     30                  35                  40
aag ctc ctg atc tat tat gca act agt ttg gca gat ggg gtc cca tca   240
Lys Leu Leu Ile Tyr Tyr Ala Thr Ser Leu Ala Asp Gly Val Pro Ser
 45                  50                  55                  60
agg ttc agt ggc tcc gga tct ggt acc gat tac act ctc acc atc tcg   288
Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Tyr Thr Leu Thr Ile Ser
                 65                  70                  75
agc ctc cag cct gaa gat att gca act tac tat tgt ctg cag cat ggt   336
Ser Leu Gln Pro Glu Asp Ile Ala Thr Tyr Tyr Cys Leu Gln His Gly
             80                  85                  90
```

gag agc ccg tac acg ttc ggc gga ggg acc aag gtg gag atc aaa 381
Glu Ser Pro Tyr Thr Phe Gly Gly Gly Thr Lys Val Glu Ile Lys
95 100 105

<210> 90
<211> 107
<212> PRT
<213> Artificial Sequence
<220>
<223> Amino acid sequence of version "c" of humanized L chain V region
<400> 90

Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
1 5 10 15
Asp Arg Val Thr Ile Thr Cys Lys Ala Ser Gln Asp Ile Lys Ser Phe
20 25 30
Leu Ser Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
35 40 45
Tyr Tyr Ala Thr Ser Leu Ala Asp Gly Val Pro Ser Arg Phe Ser Gly
50 55 60
Ser Gly Ser Gly Thr Asp Tyr Thr Leu Thr Ile Ser Ser Leu Gln Pro
65 70 75 80
Glu Asp Ile Ala Thr Tyr Tyr Cys Leu Gln His Gly Glu Ser Pro Tyr
85 90 95
Thr Phe Gly Gly Gly Thr Lys Val Glu Ile Lys
100 105

<210> 91
<211> 72
<212> DNA
<213> Artificial Sequence
<220>
<223> FR shuffling primer F2SS
<400> 91

gtctcttaag ttggttccag cagaaaccag ggaaatctcc taagaccctg atctactatg 60
caactagtaa ca 72

<210> 92
<211> 72

<212> DNA
<213> Artificial Sequence
<220>
<223> FR shuffling primer F2SA
<400> 92
tgttactagt tgcatagtag atcagggtct taggagattt ccctggtttc tgctggaacc 60
aacttaagag ac 72
<210> 93
<211> 72
<212> DNA
<213> Artificial Sequence
<220>
<223> FR shuffling primer F2XS
<400> 93
gtctcttaag ttggtatcag cagaaaccag agaaagcccc taagtccctg atctattatg 60
caactagtaa ca 72
<210> 94
<211> 72
<212> DNA
<213> Artificial Sequence
<220>
<223> FR shuffling primer F2XA
<400> 94
tgttactagt tgcataatag atcagggact taggggcttt ctctggtttc tgctgatacc 60
aacttaagag ac 72
<210> 95
<211> 381
<212> DNA
<213> Artificial Sequence
<220>
<221> sig-peptide
<222> (1)...(60)
<220>
<221> mat-peptide
<222> (61)...(381)

<223> Nucleotide sequence coding for version "b1" of humanized L chain V region

<400> 95

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atg agg gcc cct gct cag ttt ttt ggg atc ttg ttg ctc tgg ttt cca    48
Met Arg Ala Pro Ala Gln Phe Phe Gly Ile Leu Leu Leu Trp Phe Pro
-20                 -15                 -10                 -5
ggg atc cga tgt gac atc cag atg acc cag tct cca tcc tcc ctg tct    96
Gly Ile Arg Cys Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser
                 1               5                  10
gca tct gta gga gac aga gtc acc atc acg tgc aag gcc tca cag gac   144
Ala Ser Val Gly Asp Arg Val Thr Ile Thr Cys Lys Ala Ser Gln Asp
        15                  20                  25
att aaa agc ttc tta agt tgg ttc cag cag aaa cca ggg aaa tct cct   192
Ile Lys Ser Phe Leu Ser Trp Phe Gln Gln Lys Pro Gly Lys Ser Pro
    30                  35                  40
aag acc ctg atc tac tat gca act agt ttg gca gat ggg gtc cca tca   240
Lys Thr Leu Ile Tyr Tyr Ala Thr Ser Leu Ala Asp Gly Val Pro Ser
45                  50                  55                  60
agg ttc agt ggc tcc gga tct ggt acc gat tac act ctc acc atc tcg   288
Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Tyr Thr Leu Thr Ile Ser
                65                  70                  75
agc ctc cag cct gaa gat ttt gca act tac tat tgt ctg cag cat ggt   336
Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Leu Gln His Gly
            80                  85                  90
gag agc ccg tac acg ttc ggc gga ggg acc aag gtg gag atc aaa       381
Glu Ser Pro Tyr Thr Phe Gly Gly Gly Thr Lys Val Glu Ile Lys
        95                  100                 105
```

<210> 96

<211> 107

<212> PRT

<213> Artificial Sequence

<220>

<223> Amino acid sequence of version "b1" of humanized L chain V region

<400> 96

Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
1 5 10 15
Asp Arg Val Thr Ile Thr Cys Lys Ala Ser Gln Asp Ile Lys Ser Phe
20 25 30
Leu Ser Trp Phe Gln Gln Lys Pro Gly Lys Ser Pro Lys Thr Leu Ile
35 40 45
Tyr Tyr Ala Thr Ser Leu Ala Asp Gly Val Pro Ser Arg Phe Ser Gly
50 55 60
Ser Gly Ser Gly Thr Asp Tyr Thr Leu Thr Ile Ser Ser Leu Gln Pro
65 70 75 80
Glu Asp Phe Ala Thr Tyr Tyr Cys Leu Gln His Gly Glu Ser Pro Tyr
85 90 95
Thr Phe Gly Gly Gly Thr Lys Val Glu Ile Lys
100 105

<210> 97
<211> 381
<212> DNA
<213> Artificial Sequence
<220>
<221> sig-peptide
<222> (1)...(60)
<220>
<221> mat-peptide
<222> (61)...(381)
<223> Nucleotide sequence coding for version "b2" of humanized L chain V region
<400> 97

atg agg gcc cct gct cag ttt ttt ggg atc ttg ttg ctc tgg ttt cca 48
Met Arg Ala Pro Ala Gln Phe Phe Gly Ile Leu Leu Leu Trp Phe Pro
-20 -15 -10 -5
ggg atc cga tgt gac atc cag atg acc cag tct cca tcc tcc ctg tct 96
Gly Ile Arg Cys Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser
1 5 10
gca tct gta gga gac aga gtc acc atc acg tgc aag gcc tca cag gac 144
Ala Ser Val Gly Asp Arg Val Thr Ile Thr Cys Lys Ala Ser Gln Asp
15 20 25

att aaa agc ttc tta agt tgg tat cag cag aaa cca gag aaa gcc cct 192
Ile Lys Ser Phe Leu Ser Trp Tyr Gln Gln Lys Pro Glu Lys Ala Pro
30 35 40
aag tcc ctg atc tat tat gca act agt ttg gca gat ggg gtc cca tca 240
Lys Ser Leu Ile Tyr Tyr Ala Thr Ser Leu Ala Asp Gly Val Pro Ser
45 50 55 60
agg ttc agt ggc tcc gga tct ggt acc gat tac act ctc acc atc tcg 288
Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Tyr Thr Leu Thr Ile Ser
65 70 75
agc ctc cag cct gaa gat ttt gca act tac tat tgt ctg cag cat ggt 336
Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Leu Gln His Gly
80 85 90
gag agc ccg tac acg ttc ggc gga ggg acc aag gtg gag atc aaa 381
Glu Ser Pro Tyr Thr Phe Gly Gly Gly Thr Lys Val Glu Ile Lys
95 100 105

<210> 98
<211> 107
<212> PRT
<213> Artificial Sequence
<220>
<223> Amino acid sequence of version "b2" of humanized L chain V region
<400> 98

Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
1 5 10 15
Asp Arg Val Thr Ile Thr Cys Lys Ala Ser Gln Asp Ile Lys Ser Phe
20 25 30
Leu Ser Trp Tyr Gln Gln Lys Pro Glu Lys Ala Pro Lys Ser Leu Ile
35 40 45
Tyr Tyr Ala Thr Ser Leu Ala Asp Gly Val Pro Ser Arg Phe Ser Gly
50 55 60
Ser Gly Ser Gly Thr Asp Tyr Thr Leu Thr Ile Ser Ser Leu Gln Pro
65 70 75 80
Glu Asp Phe Ala Thr Tyr Tyr Cys Leu Gln His Gly Glu Ser Pro Tyr
85 90 95
Thr Phe Gly Gly Gly Thr Lys Val Glu Ile Lys
100 105

<210> 99
<211> 117
<212> PRT
<213> Mouse
<220>
<223> Amino acid sequence of H chain V region of anti TF mouse monoclonal antibody ATR-5
<400> 99

```
Glu Val Gln Leu Gln Gln Ser Gly Thr Asn Leu Val Arg Pro Gly Ala
                  5                  10                  15
Leu Val Lys Leu Ser Cys Lys Gly Ser Gly Phe Asn Ile Lys Asp Tyr
             20                  25                  30
Tyr Met His Trp Val Lys Gln Arg Pro Glu Gln Gly Leu Glu Trp Ile
         35                  40                  45
Gly Gly Asn Asp Pro Ala Asn Gly His Ser Met Tyr Asp Pro Lys Phe
     50                  55                  60
Gln Gly Lys Ala Ser Ile Thr Ala Asp Thr Ser Ser Asn Thr Ala Tyr
 65                  70                  75                  80
Leu Gln Leu Ser Ser Leu Thr Ser Glu Asp Thr Ala Val Tyr Phe Cys
                 85                  90                  95
Ala Arg Asp Ser Gly Tyr Ala Met Asp Tyr Trp Gly Gln Gly Thr Ser
            100                 105                 110
Val Thr Val Ser Ser
        115
```

<210> 100
<211> 107
<212> PRT
<213> Mouse
<220>
<223> Amino acid sequence of L chain V region of anti TF mouse monoclonal antibody ATR-5
<400> 100

```
Asp Ile Lys Met Thr Gln Ser Pro Ser Ser Met Tyr Ala Ser Leu Gly
                  5                  10                  15
Glu Arg Val Thr Ile Thr Cys Lys Ala Ser Gln Asp Ile Lys Ser Phe
             20                  25                  30
```

Leu Ser Trp Tyr Gln Gln Lys Pro Trp Lys Ser Pro Lys Thr Leu Ile
35 40 45
Tyr Tyr Ala Thr Ser Leu Ala Asp Gly Val Pro Ser Arg Phe Ser Gly
50 55 60
Ser Gly Ser Gly Gln Asp Tyr Ser Leu Thr Ile Asn Asn Leu Glu Ser
65 70 75 80
Asp Asp Thr Ala Thr Tyr Tyr Cys Leu Gln His Gly Glu Ser Pro Tyr
85 90 95
Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys
100 105

<210> 101
<211> 780
<212> DNA
<213> Homosapiens
<220>
<223> DNA coding for soluble human TF
<400> 101

atg gag acc cct gcc tgg ccc cgg gtc ccg cgc ccc gag acc gcc gtc 48
Met Glu Thr Pro Ala Trp Pro Arg Val Pro Arg Pro Glu Thr Ala Val
-30 -25 -20
gct cgg acg ctc ctg ctc ggc tgg gtc ttc gcc cag gtg gcc ggc gct 96
Ala Arg Thr Leu Leu Leu Gly Trp Val Phe Ala Gln Val Ala Gly Ala
-15 -10 -5 -1
tca ggc act aca aat act gtg gca gca tat aat tta act tgg aaa tca 144
Ser Gly Thr Thr Asn Thr Val Ala Ala Tyr Asn Leu Thr Trp Lys Ser
1 5 10 15
act aat ttc aag aca att ttg gag tgg gaa ccc aaa ccc gtc aat caa 192
Thr Asn Phe Lys Thr Ile Leu Glu Trp Glu Pro Lys Pro Val Asn Gln
20 25 30
gtc tac act gtt caa ata agc act aag tca gga gat tgg aaa agc aaa 240
Val Tyr Thr Val Gln Ile Ser Thr Lys Ser Gly Asp Trp Lys Ser Lys
35 40 45
tgc ttt tac aca aca gac aca gag tgt gac ctc acc gac gag att gtg 288
Cys Phe Tyr Thr Thr Asp Thr Glu Cys Asp Leu Thr Asp Glu Ile Val
50 55 60

aag gat gtg aag cag acg tac ttg gca cgg gtc ttc tcc tac ccg gca 366
Lys Asp Val Lys Gln Thr Tyr Leu Ala Arg Val Phe Ser Tyr Pro Ala
65 70 75 80
ggg aat gtg gag agc acc ggt tct gct ggg gag cct ctg tat gag aac 384
Gly Asn Val Glu Ser Thr Gly Ser Ala Gly Glu Pro Leu Tyr Glu Asn
85 90 95
tcc cca gag ttc aca cct tac ctg gag aca aac ctc gga cag cca aca 432
Ser Pro Glu Phe Thr Pro Tyr Leu Glu Thr Asn Leu Gly Gln Pro Thr
100 105 110
att cag agt ttt gaa cag gtg gga aca aaa gtg aat gtg acc gta gaa 480
Ile Gln Ser Phe Glu Gln Val Gly Thr Lys Val Asn Val Thr Val Glu
115 120 125
gat gaa cgg act tta gtc aga agg aac aac act ttc cta agc ctc cgg 528
Asp Glu Arg Thr Leu Val Arg Arg Asn Asn Thr Phe Leu Ser Leu Arg
130 135 140
gat gtt ttt ggc aag gac tta att tat aca ctt tat tat tgg aaa tct 576
Asp Val Phe Gly Lys Asp Leu Ile Tyr Thr Leu Tyr Tyr Trp Lys Ser
145 150 155 160
tca agt tca gga aag aaa aca gcc aaa aca aac act aat gag ttt ttg 624
Ser Ser Ser Gly Lys Lys Thr Ala Lys Thr Asn Thr Asn Glu Phe Leu
165 170 175
att gat gtg gat aaa gga gaa aac tac tgt ttc agt gtt caa gca gtg 672
Ile Asp Val Asp Lys Gly Glu Asn Tyr Cys Phe Ser Val Gln Ala Val
180 185 190
att ccc tcc cga aca gtt aac cgg aag agt aca gac agc ccg gta gag 720
Ile Pro Ser Arg Thr Val Asn Arg Lys Ser Thr Asp Ser Pro Val Glu
195 200 205
tgt atg ggc cag gag aaa ggg gaa ttc aga gaa gac tac aaa gac gat 768
Cys Met Gly Gln Glu Lys Gly Glu Phe Arg Glu Asp Tyr Lys Asp Asp
210 215 220
gac gat aaa taa 780
Asp Asp Lys
225

<210> 102
<211> 259
<212> PRT
<220>

<223> Amino acid sequence of soluble human TF

<400> 102

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Met Glu Thr Pro Ala Trp Pro Arg Val Pro Arg Pro Glu Thr Ala Val
        -30                 -25                 -20
Ala Arg Thr Leu Leu Leu Gly Trp Val Phe Ala Gln Val Ala Gly Ala
    -15                 -10                 -5                  -1
Ser Gly Thr Thr Asn Thr Val Ala Ala Tyr Asn Leu Thr Trp Lys Ser
  1               5                  10                  15
Thr Asn Phe Lys Thr Ile Leu Glu Trp Glu Pro Lys Pro Val Asn Gln
            20                  25                  30
Val Tyr Thr Val Gln Ile Ser Thr Lys Ser Gly Asp Trp Lys Ser Lys
         35                  40                  45
Cys Phe Tyr Thr Thr Asp Thr Glu Cys Asp Leu Thr Asp Glu Ile Val
     50                  55                  60
Lys Asp Val Lys Gln Thr Tyr Leu Ala Arg Val Phe Ser Tyr Pro Ala
 65                  70                  75                  80
Gly Asn Val Glu Ser Thr Gly Ser Ala Gly Glu Pro Leu Tyr Glu Asn
                85                  90                  95
Ser Pro Glu Phe Thr Pro Tyr Leu Glu Thr Asn Leu Gly Gln Pro Thr
            100                 105                 110
Ile Gln Ser Phe Glu Gln Val Gly Thr Lys Val Asn Val Thr Val Glu
        115                 120                 125
Asp Glu Arg Thr Leu Val Arg Arg Asn Asn Thr Phe Leu Ser Leu Arg
    130                 135                 140
Asp Val Phe Gly Lys Asp Leu Ile Tyr Thr Leu Tyr Tyr Trp Lys Ser
145                 150                 155                 160
Ser Ser Ser Gly Lys Lys Thr Ala Lys Thr Asn Thr Asn Glu Phe Leu
                165                 170                 175
Ile Asp Val Asp Lys Gly Glu Asn Tyr Cys Phe Ser Val Gln Ala Val
            180                 185                 190
Ile Pro Ser Arg Thr Val Asn Arg Lys Ser Thr Asp Ser Pro Val Glu
        195                 200                 205
Cys Met Gly Gln Glu Lys Gly Glu Phe Arg Glu Asp Tyr Lys Asp Asp
    210                 215                 220
Asp Asp Lys
225
```

<210> 103

<211> 780
<212> DNA
<213> Homosapiens
<220>
<223> DNA coding for human TF
<400> 103

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atg gag acc cct gcc tgg ccc cgg gtc ccg cgc ccc gag acc gcc gtc    48
MET Glu Thr Pro Ala Trp Pro Arg Val Pro Arg Pro Glu Thr Ala Val
        -30                 -25                 -20

gct cgg acg ctc ctg ctc ggc tgg gtc ttc gcc cag gtg gcc ggc gct    96
Ala Arg Thr Leu Leu Leu Gly Trp Val Phe Ala Gln Val Ala Gly Ala
    -15                 -10                 -5              -1

tca ggc act aca aat act gtg gca gca tat aat tta act tgg aaa tca   144
Ser Gly Thr Thr Asn Thr Val Ala Ala Tyr Asn Leu Thr Trp Lys Ser
  1               5                  10                  15

act aat ttc aag aca att ttg gag tgg gaa ccc aaa ccc gtc aat caa   192
Thr Asn Phe Lys Thr Ile Leu Glu Trp Glu Pro Lys Pro Val Asn Gln
            20                  25                  30

gtc tac act gtt caa ata agc act aag tca gga gat tgg aaa agc aaa   240
Val Tyr Thr Val Gln Ile Ser Thr Lys Ser Gly Asp Trp Lys Ser Lys
        35                  40                  45

tgc ttt tac aca aca gac aca gag tgt gac ctc acc gac gag att gtg   288
Cys Phe Tyr Thr Thr Asp Thr Glu Cys Asp Leu Thr Asp Glu Ile Val
    50                  55                  60

aag gat gtg aag cag acg tac ttg gca cgg gtc ttc tcc tac ccg gca   336
Lys Asp Val Lys Gln Thr Tyr Leu Ala Arg Val Phe Ser Tyr Pro Ala
65                  70                  75                  80

ggg aat gtg gag agc acc ggt tct gct ggg gag cct ctg tat gag aac   384
Gly Asn Val Glu Ser Thr Gly Ser Ala Gly Glu Pro Leu Tyr Glu Asn
                85                  90                  95

tcc cca gag ttc aca cct tac ctg gag aca aac ctc gga cag cca aca   432
Ser Pro Glu Phe Thr Pro Tyr Leu Glu Thr Asn Leu Gly Gln Pro Thr
            100                 105                 110

att cag agt ttt gaa cag gtg gga aca aaa gtg aat gtg acc gta gaa   480
Ile Gln Ser Phe Glu Gln Val Gly Thr Lys Val Asn Val Thr Val Glu
        115                 120                 125
```

```
gat gaa cgg act tta gtc aga agg aac aac act ttc cta agc ctc cgg   528
Asp Glu Arg Thr Leu Val Arg Arg Asn Asn Thr Phe Leu Ser Leu Arg
    130                 135                 140
gat gtt ttt ggc aag gac tta att tat aca ctt tat tat tgg aaa tct   576
Asp Val Phe Gly Lys Asp Leu Ile Tyr Thr Leu Tyr Tyr Trp Lys Ser
145                 150                 155                 160
tca agt tca gga aag aaa aca gcc aaa aca aac act aat gag ttt ttg   624
Ser Ser Ser Gly Lys Lys Thr Ala Lys Thr Asn Thr Asn Glu Phe Leu
                165                 170                 175
att gat gtg gat aaa gga gaa aac tac tgt ttc agt gtt caa gca gtg   672
Ile Asp Val Asp Lys Gly Glu Asn Tyr Cys Phe Ser Val Gln Ala Val
            180                 185                 190
att ccc tcc cga aca gtt aac cgg aag agt aca gac agc ccg gta gag   720
Ile Pro Ser Arg Thr Val Asn Arg Lys Ser Thr Asp Ser Pro Val Glu
        195                 200                 205
tgt atg ggc cag gag aaa ggg gaa ttc aga gaa ata ttc tac atc att   768
Cys MET Gly Gln Glu Lys Gly Glu Phe Arg Glu Ile Phe Tyr Ile Ile
    210                 215                 220
gga gct gtg gta ttt gtg gtc atc atc ctt gtc atc atc ctg gct ata   816
Gly Ala Val Val Phe Val Val Ile Ile Leu Val Ile Ile Leu Ala Ile
225                 230                 235                 240
tct cta cac aag tgt aga aag gca gga gtg ggg cag agc tgg aag gag   864
Ser Leu His Lys Cys Arg Lys Ala Gly Val Gly Gln Ser Trp Lys Glu
                245                 250                 255
aac tcc cca ctg aat gtt tca taa                                  888
Asn Ser Pro Leu Asn Val Ser ***
            260
```

<210> 104
<211> 259
<212> PRT
<220>
<223> Amino acid sequence of soluble human TF
<400> 104

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MET Glu Thr Pro Ala Trp Pro Arg Val Pro Arg Pro Glu Thr Ala Val
        -30                 -25                 -20
Ala Arg Thr Leu Leu Leu Gly Trp Val Phe Ala Gln Val Ala Gly Ala
    -15                 -10                 -5                  -1
```

Ser Gly Thr Thr Asn Thr Val Ala Ala Tyr Asn Leu Thr Trp Lys Ser
1 5 10 15
Thr Asn Phe Lys Thr Ile Leu Glu Trp Glu Pro Lys Pro Val Asn Gln
20 25 30
Val Tyr Thr Val Gln Ile Ser Thr Lys Ser Gly Asp Trp Lys Ser Lys
35 40 45
Cys Phe Tyr Thr Thr Asp Thr Glu Cys Asp Leu Thr Asp Glu Ile Val
50 55 60
Lys Asp Val Lys Gln Thr Tyr Leu Ala Arg Val Phe Ser Tyr Pro Ala
65 70 75 80
Gly Asn Val Glu Ser Thr Gly Ser Ala Gly Glu Pro Leu Tyr Glu Asn
85 90 95
Ser Pro Glu Phe Thr Pro Tyr Leu Glu Thr Asn Leu Gly Gln Pro Thr
100 105 110
Ile Gln Ser Phe Glu Gln Val Gly Thr Lys Val Asn Val Thr Val Glu
115 120 125
Asp Glu Arg Thr Leu Val Arg Arg Asn Asn Thr Phe Leu Ser Leu Arg
130 135 140
Asp Val Phe Gly Lys Asp Leu Ile Tyr Thr Leu Tyr Tyr Trp Lys Ser
145 150 155 160
Ser Ser Ser Gly Lys Lys Thr Ala Lys Thr Asn Thr Asn Glu Phe Leu
165 170 175
Ile Asp Val Asp Lys Gly Glu Asn Tyr Cys Phe Ser Val Gln Ala Val
180 185 190
Ile Pro Ser Arg Thr Val Asn Arg Lys Ser Thr Asp Ser Pro Val Glu
195 200 205
Cys MET Gly Gln Glu Lys Gly Glu Phe Arg Glu Ile Phe Tyr Ile Ile
210 215 220
Gly Ala Val Val Phe Val Val Ile Ile Leu Val Ile Ile Leu Ala Ile
225 230 235 240
Ser Leu His Lys Cys Arg Lys Ala Gly Val Gly Gln Ser Trp Lys Glu
245 250 255
Asn Ser Pro Leu Asn Val Ser
260

DEPOSITOR
Name: CHUGAI SEIYAKU
KABUSHIKI KAISHA

Representative: Osamu Nagayama

Address: 5-1, Ukima 5-chome
Kita-ku, Tokyo

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT
issued pursuant to Rule 7.1 by the INTERNATIONAL DEPOSITARY AUTHORITY identified at the bottom of this page.

I. IDENTIFICATION OF THE MICROORGANISM

Identification reference given by the DEPOSITOR: KPMM2

(Deposition Number)
FERM BP-7419

II. SCIENTIFIC DESCRIPTION AND/OR TAXONOMIC DESIGNATION

The microorganism identified under I above was accompanied by:

× a scientific description

× a proposed taxonomic designation

(Mark with a cross where applicable)

III. RECEIPT AND ACCEPTANCE

This International Depositary Authority accepts the microorganism identified under I above, which was received by it on February 22, 1994 (Date of the original deposit)[1].

IV. RECEIPT OF TRANSFER

This International Depositary Authority accepted the microorganisms identified under I above, on February 22, 1994 (Date of the original deposit), and accepted a request for transfer to a deposition under Budapest treaty from the original deposition, on December 27, 2000. (Transferred from FERM P-14170 deposited on February 22, 1994.)

V. INTERNATIONAL DEPOSITARY AUTHORITY

National Institute of Science and Human-Technology

Agency of Industrial Science and Technology

Director General Shinichi Ohashi

1-3, Higashi 1 chome, Tsukuba-shi,
Ibaraki-ken, 305-8566, Japan

December 27, 2000

国際様式　INTERNATIONAL FORM

[特許手続上の微生物の寄託の国際的承認に関するブダペスト条約]

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE

下記国際寄託当局によって規則７．１に従い発行される。

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT

issued pursuant to Rule 7.1 by the INTERNATIONAL DEPOSITARY AUTHORITY identified at the bottom of this page.

原寄託についての受託証

寄託者
氏名（名称）　中外製薬株式会社
取締役社長　永山　治　殿
あて名　〒
東京都北区浮間５丁目５番１号

１．微生物の表示	
（寄託者が付した識別のための表示） KPMM2	（受託番号） FERM BP－ 7419

２．科学的性質及び分類学上の位置

１欄の微生物には、次の事項を記載した文書が添付されていた。

■ 科学的性質
■ 分類学上の位置

３．受領及び受託

本国際寄託当局は、平成　６年　２月２２日（原寄託日）に受領した１欄の微生物を受託する。

４．移管請求の受領

本国際寄託当局は、平成　６年　２月２２日（原寄託日）に１欄の微生物を受領した。
そして、平成１２年１２月２７日に原寄託よりブダペスト条約に基づく寄託への移管請求を受領した。
（平成　６年　２月２２日に寄託された微工研菌寄第Ｐ－　１４１７０　号より移管）

５．国際寄託当局

通商産業省工業技術院生命工学工業技術研究所

名称：　National Institute of Bioscience and Human-Technology
Agency of Industrial Science and Technology

所長　大箸　信一
Dr. Shinichi Ohashi Director-General

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305-8566, JAPAN

平成１２年（２０００）１２月２７日